ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

ANTIOXIDATIVE EFFECTS OF *FUCUS VESICULOSUS* EXTRACTS IN FISH OIL ENRICHED MAYONNAISE

M.Sc. THESIS

Betül YEŞİLTAŞ

Department of Food Engineering

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

FUCUS VESICULOSUS EKSTRAKTLARININ BALIK YAĞI İLE ZENGİNLEŞTİRİLMİŞ MAYONEZ ÜZERİNDEKİ ANTİOKSİDATİF ETKİLERİ

YÜKSEK LİSANS TEZİ

Betül YEŞİLTAŞ
(506111507)

Gıda Mühendisliği Anabilim Dalı

Gıda Mühendisliği Programı

Tez Danışmanı: Yard. Doç. Dr. Esra ÇAPANOĞLU GÜVEN

TEMMUZ 2013

Betül Yeşiltaş, a **M.Sc.** student of ITU **Institute of Science and Technology/Graduate School of Istanbul Technical University** student ID 506111507, successfully defended the **thesis** entitled "**ANTIOXIDATIVE EFFECTS OF *FUCUS VESICULOSUS* EXTRACTS IN FISH OIL ENRICHED MAYONNAISE**", which she prepared after fulfilling the requirements specified in the associated legislations, before the jury whose signatures are below.

Thesis Advisor : **Asst. Prof. Dr. Esra ÇAPANOĞLU GÜVEN** .....
İstanbul Technical University

Co-advisor : **Öğr. Gör. Dr. Ebru FIRATLIGİL DURMUŞ** .....
İstanbul Technical University

Jury Members : **Asst. Prof. Dr. Esra ÇAPANOĞLU GÜVEN** .....
İstanbul Technical University

Öğr. Gör. Dr. Ebru FIRATLIGİL DURMUŞ .....
İstanbul Technical University

Prof. Dr. Beraat ÖZÇELİK .....
İstanbul Technical University

Prof. Dr. Melek TÜTER .....
İstanbul Technical University

Asst. Prof. Dr. Derya KAHVECİ .....
Yeditepe University

Date of Submission : 01 July 2013
Date of Defense : 17 July 2013

To my family,

FOREWORD

This thesis was prepared in the period 2nd of January to 30th of June at the National Food Institute at Technical University of Denmark.

I would like to express my thanks to my supervisors Charlotte Jacobsen, Ditte Baun Larsen, Esra Çapanoğlu Güven, Ebru Fıratlıgil Durmuş and people in the DTU lipid group for being helpful and suportive during my study.

I am also grateful to my family and all my friends both living in Turkey and Denmark.

July 2013

Betül YEŞİLTAŞ
(Food Engineer)

TABLE OF CONTENTS

Page

ABBREVIATIONS

F. vesiculosus	: *Fucus vesiculosus*
WE	: Water Extract
EAE	: Ethylacetate Extract
TPC	: Total phlorotannin content
Mayo	: Mayonnaise
DF	: Diol Fraction
AF	: Amino Fraction
PUFA	: Polyunsaturated fatty acid
DHA	: Docosahexaenoic acid
EPA	: Eicosapentaenoic acid
LH	: Unsaturated lipid
L*	: Radical-alkyl
LOO*	: Peroxides radical
LOOH	: Hydroperoxide
PV	: Peroxide value
FFA	: Free fatty acids
O/W	: Oil in water emulsions
DHS	: Dynamic head space
GC-MS	: Gas chromatography–mass spectrometry
LC-MS	: Liquid Chromatography-mass spectrometry
FAME	: Fatty acid methyl ester method
GC- FID	: Gas chromatography- flame ionization detection
HPLC-FLD	: High pressure liquid chromatography-flourescence detection
HPLC-DAD	: High pressure liquid chromatography-diode array detectors
PG	: Post gastric fraction of bioavailability
IN	: Samples taken from inside of the dialysis tube
OUT	: Samples taken from outside of the dialysis tube

LIST OF TABLES

Page

LIST OF FIGURES

Page

ANTIOXIDATIVE EFFECTS OF *FUCUS VESICULOSUS* EXTRACTS IN FISH OIL ENRICHED MAYONNAISE

SUMMARY

During the recent decade, there has been an increasing demand for consuming omega-3 enriched products. Many studies have reported that omega-3 fatty acids, especially EPA and DHA, have health promoting effects. However, consumption of these compounds, which can be found in fish and fish products such as fish oil, is under the recommended daily intake (RDI) numbers. In order to increase the consumption of omega-3 fatty acids, mayonnaise, which consists of 80% vegetable oil, was chosen as a food system to be enriched. Mayonnaise was produced by substituting 20% of vegetable oil (rape seed oil) with fish oil. As known, fish oil is extremely prone to lipid oxidation due to the fact that it contains polyunsaturated fatty acids such as EPA and DHA which have 5 and 6 double bonds, respectively. In this thesis, brown algae *Fucus vesiculosus* extracts were used as an antioxidant in order to see the effects of them on oxidation stability in fish oil enriched mayonnaise. *F. vesiculosus* contains high amounts of phenolic compounds called "phlorotannins". Preliminary characterization of phlorotannins in water and ethylacetate extracts were done by LC-MS and identified for instance; fucophloroethol A, fucodiphloroethol G, fucotriphlorethol A, trifucodiphlorethol A. Total phlorotannin content of water and ethylacetate extracts of *F. vesiculosus* were found to be 28.5 and 45.3 g PGE/ 100 g, respectively and these extracts were found to have antioxidative activity in *in vitro* antioxidant assays such as DPPH radical scavenging activity, reducing power and metal chelating activity. Water and ethylacetate extracts of *F. vesiculosus* were digested through *in vitro* gastrointestinal system and bioavailability of water and ethylacetate extracts were found to be 5.9% and 6.8%, respectively. Different concentrations (1, 1.5 and 2 g/kg) of water and ethylacetate extracts of *F. vesiculosus* were added to fish oil enriched mayonnaise and the effects on oxidation stability were evaluated for each sample compared to a reference mayonnaise without antioxidant during 4 weeks storage period at 20 ^{o}C. Peroxide value results indicated that the highest antioxidant activity was shown by water extract when added in a concentration of 2 g/kg. Findings suggested that, mayonnaise samples with smaller droplet sizes were oxidised faster at the initial part of the storage period than the ones with larger droplet sizes. Samples with *F. vesiculosus* extracts were found to have an protective effect on total tocopherol content compared to reference sample. Additionally, fatty acid composition data showed that water and ethylacetate extracts of *F. vesiculosus* had an effect on preventing EPA and DHA loss during storage. Water extract of *F. vesiculosus* added in a concentration of 2 g/kg had an effect on preventing the formation of volatile oxidation products such as pentanal, 1-penten-3-ol, 3-methyl-1-butanol.

FUCUS VESICULOSUS EKSTRAKTLARININ BALIK YAĞI İLE ZENGİNLEŞTİRİLMİŞ MAYONEZ ÜZERİNDEKİ ANTİOKSİDATİF ETKİLERİ

ÖZET

Son yıllarda, omega-3 ile zenginleştirilmiş gıdaların tüketiminin insan sağlığı üzerine faydaları üzerine araştırma yapılmakta ve tüketiminin yaygınlaştırılması sağlanmaktadır. Bir çok çalışma omega-3 yağ asitlerinin, özellikle EPA ve DHA olmak üzere, sağlığı olumlu yönde etkileyen özelliklere sahip olduğunu kanıtlamıştır.

Ancak, istatistiksel verilere bakıldığında geniş bir coğrafyada bu önemli yağ asitlerinin tüketimi günlük önerilen tüketim değerinin altında olduğu açıkça görülmektedir. Dolayısıyla EPA ve DHA yağ asitlerinin tüketimini arttırmak amacıyla balık ve balık yağı türevlerinin tüketimini arttırıcı yönde öneriler sunan çalışmalar önem kazanmakadır.

Özellikle, yağ içeriği yüksek bazı gıda ürünleri, balık yağı ile zenginleştirilerek, yine EPA ve DHA omega-3 yağ asitlerinin tüketimi arttırılmaya çalışılmaktadır. Örneğin, mayonez ürünü içerdiği yüksek yağ oranı ile (%80) balık yağı ile zenginleştirmeye uygun bir ürün olarak görülmüştür.

Bu çalışmada ise bitkisel yağ (kanola yağı) içeriği % 80 olan mayonez ürünü ele alımış ve balık yağı ile zenginleştirilmiştir. Kanola yağının %20 si balık yağı ile değiştirilerek mayonezin EPA ve DHA omega-3 yağ asidi bileşenleri içermesi sağlanmıştır. Ancak bilindiği üzere, balık yağı oksidasyona oldukça hassas bir üründür. Bunun sebebi, EPA ve DHA gibi yapısında sırasıyla 5 ve 6 adet çift bağ bulunan çoklu doymamış yağ asitlerini bileşiminde içermesidir. Dolayısıyla, balık yağı ile zenginleştirilmiş bir ürünün uygun bir antioksidan madde ile desteklenerek, oksidasyona hassas olan bu değerli omega-3 yağ asitleri korunmalı ve tüketiciye yararlanılabilecek birer bileşik olarak ulaştırılmalıdır.

Bu çalışmada, kahverengi yosun olan *Fucus vesiculosus'*un su ve etilasetat ekstaktları, doğal antioksidan olarak kullanılarak, balık yağı ile zenginleştirilmiş mayonez üzerindeki antioksidatif stabiliteye etkileri incelenmiştir. *F. vesiculosus*'un su ve etilasetat ekstarktları içerisinde bulunan, "florotanin" olarak adlandırılan fenolik maddenin güçlü bir atioksidan madde olduğu üç farklı antioksidan metodu kullanılarak saptanmıştır.

Ayrıca ekstraktların antioksidan etki göstermesini sağlayan temel fenolik bileşenlerin yani florotaninlerin fraksiyonlarına ayrılması katı faz ekstraksiyonu yöntemi kullanılarak, Oasis Max, Amino ve Diol hazır kolonları kullanılarak yapılmıştır. Fraksiyonlarına ayırma işlemi sırasında kullanılan farklı polaritedeki çözgelerin farklı miktarlarda karıştırılması ile polaritede değişim sağlanmıştır. Aynı zamanda çözgen karışımlarının pH ı değiştirilerek florotaninlerin polaritelerine göre farklı fraksiyonlarına ayrılması sağlanmıştır.

Elde edilen fraksiyonların hepsi LC-MS kullanılarak incelenmiştir, sadece diol kolonundan elde edilen fraksiyonlarda bilinen florotanin molekül ağırlıklarına rastlanmıştır ve karakterizasyonu yapılmıştır. Bu ön karakterizasyon işlemi sonucunda dört farklı molekül ağırlığına sahip florotanin tespit edilmiştir. Bu florotaninler hem su hem de etilasetat ekstraktları içinde bulunmuştur: fucophloroethol A, fucodiphloroethol G, fucotriphlorethol A, trifucodiphlorethol A.

Antioksidan aktivitesinin doğrudan etkilendiği bilinen *F. vesiculosus* ekstraktlarının toplam florotanin içeriği de çalışma kapsamında incelenmiştir. *F. vesiculosus'*un su ve etilasetat ekstraktlarının tespit edilen toplam florotanin içeriği sonuçları sırasıyla 28.5 and 45.3 g PGE/ 100 g olarak literatür ile de uyumlu bir şekilde bulunmuştur.

Antioksidan aktiviteleri de ayrıca tespit edilen *F. vesiculosus* ekstraktları, DPPH radical yakalama aktivitesi, indirgeme potansiyeli ve metal iyonu şelatlama aktivitesi metotları kullanılarakanalizlenmiştir. Sonuçlar incelendiğinde *F. vesiculosus* ekstraktlarının güçlü antioksidan aktiviteye sahip oldukları gözlenmiştir.

Antioksidan aktivite açısından incelendiğinde balık yağı ile zenginleştirilmiş mayonez için en önemli aktivite metal şelatlama aktivitesi olduğu önceden yapılan araştırmalarda belirtilmiştir. Bunun sebebi ise mayonez yapımında kullanılan yumurta akı ve son ürünün düşük pH (pH 4) içermesidir. Çünkü düşük pH varlığında yumurta akında bağlanmış olan metaller çözülmekte ve oksidasyonu tetiklemektedirler. Dolayısıyla oksidasyonu engellemek için kullanılan antioksidan maddenin iyi bir metal şelatlama ajanı olması gerekmektedir. Yapılan çalışmada *F. vesiculosus'*un sulu ekstraktlarının oldukça iyi metal şelatlama aktivitesi olduğu gözlenmiştir.

Bu çalışmalara ek olarak, *F. vesiculosus*'un su ve etilasetat ekstraktlarının *in-vitro* metotla gastrointestinal bir sistemdeki emilimi incelenmiştir. Örneğin, toplam florotanin içeriğinin sırasıyla su ve etilasetat ekstraktları için ince bağırsağı temsil eden diyaliz tüpünden emilme yüzdesi 5.9 ve 6.8 'dir. Bu değerler literatür ile karşılaştırıldığında, ekstraktların ince bağırsağı temsil eden diyaliz tüpünden iyi bir randa emilime uğradığı yani iyi bir emilim oranına sahip olduğu söylenebilir.

F. vesiculosus 'un su ve etilasetat ekstraktlarının farklı konsantrasyonları (1, 1.5 and 2 g/kg) balık yağı ile zenginleştirilmiş mayoneze eklenerek 4 hafta 20^{o}C'de karanlıkta depolanmıştır. Sonuçlar ekstrakt içermeyen referans ürün ile farklı konsantrasyonlardaki *F. vesiculosus* sulu ve etil asetat ekstraktları ile muamele edilen ürünler ile karşılaştırılarak, oksidasyon stabilitesi üzerine etkileri incelenmiştir.

Birincil oksidasyon ürünlerinin miktarının belirlenmesinde kullanılan peroksit sayısı analizi sonuçlarına göre en yüksek antioksidatif etki gösteren mayonez ürünü, 2 g/kg su ektraktı içeren mayonez ürünü olarak belirlenmiştir. Bu sonuca dayanarak ekstraktların belirli bir konsantrasyonda diğerlerine göre daha etkin olduğunu söyleyebiliriz.

Damlacık büyüklüğü analizi sonuçları incelendiğinde, *F. vesiculosus*'un sulu ve etilasetatlı ekstraktlarının balık yağı ile zenginleştirilmiş mayoneze eklenen miktarları, diğer bir değişle ürün içindeki konsantrasyonları arttıkça damlacık büyüklüğünün de arttığı gözlenmiştir. Burada ekstraktların oluşturulan yağ-su emülsiyonları içerisinde damlacık büyüklüğünü doğrudan etkileyebileceği sonucuna varılmıştır. Ayrıca sonuçlar göstermektedir ki küçük damla boyutuna sahip olan mayonez ürünleri, depolamanın ilk döneminde (ilk hafta) geniş damla boyutuna sahip mayonez ürünlerine oranla daha hızlı okside olmaktadır. Fakat bu etki

depolamanın ileriki günlerinde (2. Hafta ile 4. Hafta sonunda) görülmemiştir. Bu bulgu, balık yağı ile zenginleştirilmiş mayonez üzerine yapılan diğer çalışmalarla da desteklenmektedir.

F. vesiculosus ekstraktlarının toplam tokoferol içeriği üzerinde koruyucu etki gösterdiği gözlemlenmiştir. Referans örneği, yani *F. vesiculosus* ekstraktarının eklenmediği balık yağı ile zenginleştirilmiş mayonez örneğinde depolamanın başlangıcı ile sonundaki toplam tokoferol içeriği kıyaslandığında, dört haftalık depolama işlemi sonucunda tocoferol içeriğinde bir azalma görülmektedir. Ancak *F. vesiculosus*'un sulu ve etilasetatlı ekstraktlarındaki başlangıç ve depolama sonucu olan değişimler incelendiğinde toplam tokoferol içeriğinde kayıp olmadığı görülmüştür.

Buna ilave olarak, tek tek alfa, beta, gama ve delta tokoferol içerikleri de incelenmiştir ve referans üründe alfa ve gama tokoferolde azalma olduğu gözlemlenirken, *F. vesiculosus* ekstraktı ile desteklenen ürünlerde herhangi bir kaybın olmadığı açıkça gözlemlenmiştir.

Daha önceki çalışmalarda tokoferol içeriğinin balık yağı ile zenginleştirilmiş mayonez ürünün içerisindeki miktarı 590 mg/kg yağ değerinden fazla olması durumunda prooksidatif etki gösterdiği görülmüştür. Bu çalışmada üretilen mayonez örnekleri bu değerin altında toplam tokoferol içeriğine sahip oldukları için tokoferollerin ürün içerisinde herhangi bir prooksidatif etki göstermedikleri tespit edilmiştir.

Çalışmada elde edilen önemli bulgulardan bir diğeri ise, yağ asidi kompozisyonundaki değişimler incelendiğinde elde edilen sonuçlardır. *F. vesiculosus* ekstraktlarının EPA ve DHA omega-3 yağ asitleri üzerinde dört hafta süresince oda sıcaklığında (20°C) ve karanlıkta depolama sonunda balık yağı ile zenginleştirilmiş mayonez üzerinde koruyucu etki gösterdiğini gözlemlenmiştir.

F. vesiculosus ekstraktlarıyla desteklenen mayonez örnekleri, ektrakt eklenmemiş referans örnek ile karşılaştırıldığında, depolama süresi boyunca elde edilen EPA ve DHA omega-3 yağ asitleri miktarlarındaki azalma en fazla referans örnekte gözlenmiştir. Bu sonuç, antioksidan özellik gösteren *F. vesiculosus* ekstraklarının her ikisinin de (sulu ve etilasetatlı ekstraktları) EPA ve DHA omega-3 yağ asitlerini koruma açısından oldukça etkili olduğunu göstermektedir.

Referans örneğinin EPA omega-3 yağ asidi değeri dört haftalık depolama sonunda %6,7 azalma gösterirken *F. vesiculosus*'un sulu ekstraktlarında bu azalım %1,7'lere, *F. vesiculosus*'un etilasetatlı ekstraktlarında ise % 0,8'lere varan azalım gözlenmiştir. DHA omega-3 yağ asidi sonuçlarındaki değişimlere bakıldığında ise; referans örneğinde dört haftalık depolama sonucu tespit edilen azalma % 14,9 iken, *F. vesiculosus*'un sulu ekstraktı kullanıldığında bu azalım %2.9 lara, *F. vesiculosus*'un etilasetatlı ekstraktları kullanıldığında ise bu değer %2,2'lere dek varmaktadır. Dolayısıyla, *F. vesiculosus*'un sulu ve etilasetatlı ekstraktları EPA ve DHA omega-3 yağ asitlerinin oksidasyonunu engellemede önemli bir rol üstlenerek, başlangıçta balık yağından elde edilen değerlerini kaybetmeden korumalarını sağlamaktadır. Bu da hem daha sağlıklı bir depolama sürecini hem de korunan besin değeri ile kaliteli bir ürün sunmada katkı sağlamaktadır.

2 g/kg konsantrasyonundaki *F. vesiculosus* sulu ekstraktının, mayonez ürününde oluşan pentanal, 1-penten-3-ol, 3-methyl-1-butanol gibi uçucu bileşiklerin oluşumunu önemli derecede engelleyici etkisi olduğu da gözlemlenmiştir.

1. INTRODUCTION

The intake of long chain n-3 polyunsaturated fatty acids (PUFA), which must be acquired through diet, is low when it is compared with the daily adequate intake (AI). The AI for α-linolenic acid is 1.6 and 1.1 g/d for men and women, respectively. The main sources of long chain n-3 PUFA in the human diet are marine fish and fish oils. Although consumption of fish and fish oil have been increasing, it is still insufficient for a healthy life.

There are number of scientific studies about consumption of fish and/or fish oil which show their health effects, especially associated with their content of long-chain polyunsaturated fatty acids (n-3 LC PUFA), most notably docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA).

Long chain n-3 PUFA have been recognised to prevent several disease such as cardiovascular, high blood pressure, hypertension, depression, Alzheimer's disease and Parkinson's disease (Tanskanen et al., 2001; Morris et al., 2003; (Hooper et al. 2006); Virtanen et al., 2008). DHA and EPA have been shown in several studies to lower the risk of several lifestyle related diseases.

In order to increase the intake of long chain n-3 PUFA, mayonnaise was enriched with fish oil due to its high content of fat which can be substituted with different amounts of fish oil. Mayonnaise as a food product includes a high amount of oil (70-80%) and a low pH (around 4) compared to other food systems (Jacobsen 2010). The oil content of mayonnaise makes lipid oxidation a major problem for mayonnaise manufacturers due to the effect of limiting shelf life of the products. Especially when the mayonnaise is enriched with fish oil, which is a good source of omega-3 fatty acids, it is particularly prone to lipid oxidation. One of the main problems associated with incorporating polyunsaturated lipids into foods is their high susceptibility to oxidation.

Studies have shown that phlorotannins derived from brown seaweeds are potent ferrous ion chelators and free radical scavengers due to their phenolic structure that includes up to eight interconnected rings of phloroglucinol which acts as an electron

trap to scavenge reactive oxygen species (ROS) (Ahn et al., 2007; Kim et al., 2009). Brown seaweeds such as *F. vesiculosus* have known with their high total phenolic content and also shown higher antioxidant activity compared with other red and gren seaweeds that had lower phenolic content (Wang et al., 2009; Farvin and Jacobsen, 2012).

With the aim of preventing lipid oxidation in fish oil enriched mayonnaise, phlorotannins which were extracted from brown seaweed (*F. vesiculosus*) and known as a strong antioxidant were researched in this study. Phlorotannins have a unique molecular structure which may be the reason for their strong antioxidant activity (Ahn et al, 2007). Phlorotannins are also natural antioxidants which are preferred by the consumers recently.

Moreover, it is also of critical importance to evaluate the effects of antioxidants that are available in the human gastrointestinal system besides the investigation of antioxidant potential of *F. vesiculosus* extracts. Thus, one of the objectives of this study was to investigate the total phenolic content and antioxidant activities of samples taken after an *in vitro* gastrointestinal system was applied to the *F. vesiculosus* extracts.

To be able to have a better understanding of lipid oxidation mechanisms, natural antioxidant usage is needed to be developed in emulsion food systems such as mayonnaise. Therefore, it is important to investigate the antioxidative effects and behaviours of phlorotannins in an oil-in-water emulsion which is prone to lipid oxidation.

1.1 Purpose of Thesis

This thesis is a part of a PhD project with the main subject: Extraction and characterization of highly bioactive ingredients from Nordic marine algae.

Phlorotannins extracted from brown algae *F. vesiculosus* were investigated in order to use as a natural antioxidant in fish oil enriched mayonnaise.

Objectives of this study are:

- Simple characterization of purified extracts and fractions of *F. vesiculosus*
- To determine total phlorotannin content of *F. vesiculosus* extracts
- To assess antioxidant activity of *F. vesiculosus* extracts using DPPH radical scavenging, reducing power and ferreus ion chelating assays

- Investigating bioavailability of phlorotannins extracted from *F. vesiculosus* by using *in vitro* gastrointestinal digestion system. Taking samples from different parts of the system and determining their antioxidant activity and total phlorotannin content.
- Evaluating the antioxidant properties of crude extracts in fish oil enriched mayonnaise with these analysis:
    - peroxide value,
    - volatile oxidation compounds using DHS by GC-MS,
    - tocopherol content,
    - fatty acid composition,
    - simple sensory analysis.

1.2 Literature Review

1.2.1 Phlorotanins as an antioxidant content

Recently there is an increasing trend in using natural antioxidants instead of using synthetic antioxidants as a food additive (Wang et al, 2012). Due to their health risks and toxicity, synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, *tert* butylhydroxyquinone and propyl gallate, are strictly regulated as a food additive in many countries in order to protect against potential health hazards like carcinogenic effects (Matanjun et al. 2008, Wang et al. 2009).

Seaweeds are consumed in a relatively limited part of the world as a whole food (Cornish and Garbary, 2010). Japanese people consume seaweeds on average, 1.6 kg dry weight per person, per year (Chandini et al, 2008). Antioxidants extracted from marine macroalgae sources are also becoming important in the food industry due to consumer demands for natural food ingredients (Wang et al, 2009). Marine macroalgae such as *Fucus serratus, F. vesiculosus, F. distichus, F. spiralis, Sargassum muticum, Saccharina latissima, Laminaria digitata, Dictyota dichotoma, Enteromorpha intestinalis, Ulva lactuca, Palmaria palmata, Porphyra purpurea, Chondrus crispus, Mastocarpus stellatus, Polysiphonia fucoides,* and *Gracilaria vermiculophylla* include different amounts of phenolic compounds which have antioxidant activities (Farvin and Jacobsen, 2013).

Phlorotannins, which are phenolic compounds extracted from brown algae have antioxidant activity. In this study water and ethylacetate extracts of brown algae *F.*

vesiculosus which also includes phlorotannins will be used to see the antioxidative effects on fish oil enriched mayonnaise.

1.2.1.1 Phlorotannins

Phlorotannins which are polymers of phloroglucinol are a kind of tannins under the group of phenolic compounds. They have been identified from several brown algal families such as Alariaceae, Fucaceae and Sargassaceae (Wang et al, 2009). Previous studies have shown that the only phenolic group in brown algae (*Phaeophyceae*) is phlorotannins (Jormalainen and Honkanen, 2004; Koivikko et al, 2007). The molecular weight of these compounds are ranging from 126 to >1x10^5 Da (Parys et al, 2007).

Phlorotannins has a unique molecular structure which may have been the reason of their strong antioxidant activity reported by the previous studies (Ahn et al, 2007). Phlorotannins are categorized according to their bond types and hydroxyl groups. (Amsler and Fairhead, 2006; Steevensz et al, 2012).

1.2.1.2 Molecular structures of phlorotannins

Phlorotannins are phloroglucinol based compounds and biosynthetized by the acetate –malonate pathway, also known as the polyketide pathway with a wide range of molecular sizes (Wang et al, 2012; Amsler and Fairhead, 2006**).** As triketide has not a stable structure and tend to be altered to a phloroglucinol which has a thermodynamically more stable aromatic form including three phenolic hydroxyl groups as it is shown in Figure 1.1(Waterman and Mole, 1994; Koivikko, 2008).

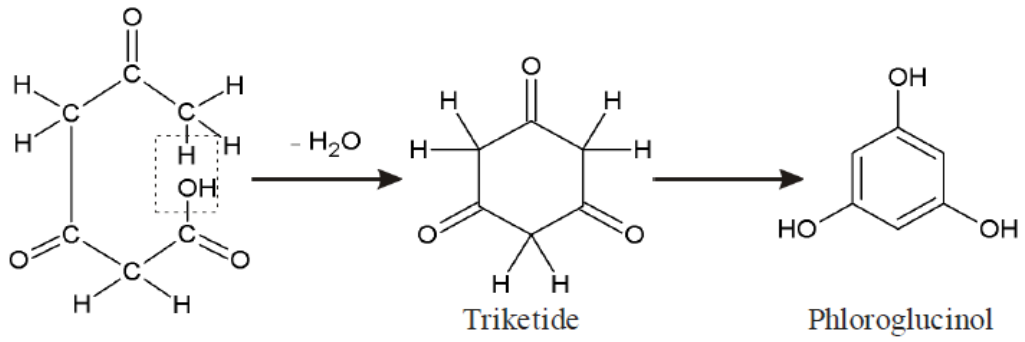

Figure 1.1: Cyclization of a triketide chain to form a phloroglucinol (adapted from Waterman and Mole, 1994; Koiviko, 2008).

Phenol rings are the main reason for the antioxidant activity of polyphenols due to their ability to behave like electron traps to scavenge peroxy, superoxide-anions and

hydroxyl radicals (Wang et al, 2009). Phlorotannins, purified from brown algae have up to eight phenol rings which can be also called as phloroglucinol units, have more potential to show antioxidant activity than other polyphenols which have less phenol rings (Wang et al, 2009). Shibata et al (2008) also reported that phlorotannins (eckol, dieckol, phlorofucofuroeckol A and 8,8`-bieckol) isolated from the Japanese brown algae *Eisenia bicyclis, Ecklonia cava and E. kurome* have shown 2-10 times more antioxidant activities as compared to catechin, α-tocopherol and ascorbic acid.

There are different types of phloroglucinols such as fucols which have a aryl-aryl bonds, fucophlorethols bonded by ether or aryl-aryl bonds and phlorethols with ether bonds (Parys et al, 2010). Figure 1.2 shows molecular structures of some of the phlorotannins.

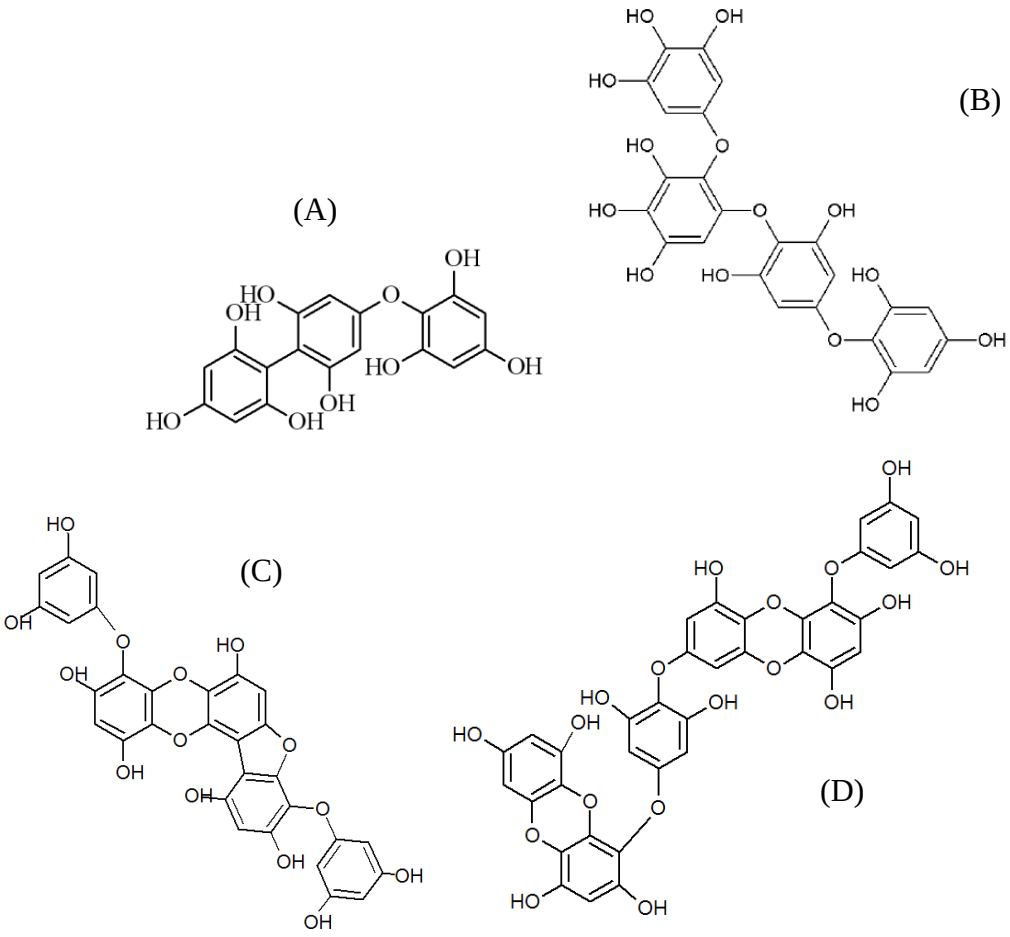

Figure 1.2: Some of the phlorotannin's molecular structures (A) Fucophloroethol A, $C_{18}H_{14}O_9$, MW: 374.29 g/mol (Ferreres et al, 2012; Liu and Gu, 2012), (B) Tetrafuhalol A, $C_{24}H_{18}O_{14}$, MW: 530 (Koivikko, 2008), (C) Phlorofucofuroeckol A, $C_{30}H_{18}O_{14}$, MW: 602.45 g/mol (Li et al, 2011), (D) dieckol, $C_{36}H_{22}O_{18}$, MW: 742.52 g/mol (Li et al, 2011).

Liu and Gu (2012) studied with the brown algae *F. vesiculosus* and isolated fucophlorethol A, tetrafucol A, trifucodiphlorethol A from *Fucus vesiculosus*. In a different study, 16 individual components of the phenolic extract of *F. vesiculosus* were separated using different gradient programs for reversed and normal phase HPLC methods such as normal phase conditions with a silica stationary phase and a mobile phase with a linear gradient of increasing polarity (Koivikko et al, 2007).

Parys et al (2010) identified phlorotannins such as trifucodiphlorethol A, trifucotriphlorethol A and fucotriphloroethol A which were separated by RP-HPLC and elucidated mainly based on NMR spectra. Another study identified fucophlorethol, fucodiphlorethol, fucotriphlorethol, 7-phloreckol, phlorofucofuroeckol and bieckol/dieckol (Ferreres et al, 2012).

1.2.2 *Fucus vesiculosus*

F. vesiculosus is a brown seaweed which is characteristic in the coasts of the North Sea, the western Baltic Sea and the Atlantic and Pacific Oceans. *F. vesiculosus* is a member of the family Fucaceae (Parys et al, 2010). *F. vesiculosus* is wide spread at the bottom layer of the Baltic Sea and dominates shallow macroalgaes. Since it is the only fucoid species, it penetrates all the way into the Gulf of Bothnia in the north and to the Gulf of Finland in the east (Torn et al, 2006).

F. vesiculosus is one of the richest sources of phlorotannins and also has the highest Fe^{2+} chelating activity among 16 species of brown seaweeds collected from Danish coasts (Farvin and Jacobsen, 2013). Wang et al (2009) showed that *F. vesiculosus* had the highest total phlorotannin content and antioxidant activity among 10 Icelandic seaweeds.

1.2.2.1 *F. vesiculosus* extracts and fractions

Koivikko et al (2005) showed that the most efficient extractant to select the phlorotannins from *F. vesiculosus* was 70 % aqueous acetone among eight different extractants which were ethyl acetate, ethanol, methanol, acetone, 70% aqueous acetone, 80% aqueous ethanol, 80% aqueous methanol, water. If only water was used as an extractant, water-soluble polysaccharides, proteins and organic acids were also extracted which is not preferable in the extract.

Wang et al (2009) also extracted *F. vesiculosus* samples using 70% aqueous acetone (v/v) and mentioned that acetone was more efficient than water to extract

polyphenols. Ferreres et al (2012) also studied the extraction efficacy 70% aqueous acetone. Steevensz et al (2012) extracted phlorotannins from *F. vesiculosus* using methanol and dichloromethane and fractionated them with a C18 Sep-Pak cartridge using methanol as a solvent.

Liu and Gu (2012) extracted phlorotannins with 70% acetone-water and fractionated with dichloromethane, ethylacetate, butanol and water, respectively. Then ethylacetate fraction was fractionated by Sephadex LH-20 and 4 subfractions were obtained. Among these fractions, ethylacetate fraction showed the highest phlorotannin concentration and 4 ethylacetate subfractions showed higher total phlorotannin content than the original *F. vesiculosus* acetone extract.

Although Wang et al (2012) compared the best extraction efficiencies of different polar solvent systems and found that acetone has the most efficient one, 80% ethanol was chosen for the fractionation of phlorotannins in order to extract food-grade natural antioxidants and also to be used as it is a food-grade alcohol.

Acetone in the extractant may have increased the total yield by inhibiting interactions between tannins and proteins during extraction or even by breaking hydrogen bonds between tannin-protein complexes (Koivikko, 2008). Parys et al (2007; 2010) extracted phlorotannins from *F. vesiculosus* using 96% ethanol in order to determine polyphenols.

Even though acetone and methanol were found as the most efficient solvent, because of safety concerns regarding the use of some organic solvent extracts in food, water and ethylacetate extracts of *F. vesiculosus* were used which were approved food-grade solvents such as foodgrade alcohol and ethyl acetate, in the current study (Wang et al., 2009).

1.2.2.2 Characterization of *F. vesiculosus* extracts and fractions

It is suitable to identificate and quantitate phlorotannins which has a high solubility in water and/or organic solvents by high performance liquid chromatography (HPLC) (Koivikko et al, 2007). Steevensz et al (2012) also mentioned that chromatographic techniques are a preferable option to analyse phlorotannins due to their high complexity and susceptibility to oxidation (Koivikko et al, 2007). Quantification of phlorotannins is difficult due to their large size of molecular structures and reactivity with other compounds (Koivikko, 2008).

Koivikko et al (2007) and Steevensz et al (2012) indicated that pH should be increased to enhance the negative mode ionisation to detect phlorotannins, which are weak acids, by NPLC.

Reversed Phase (RP) HPLC was not considered as an appropriate choice due to the high polarity of phlorotannins which results in lack of interaction with non-polar stationary phase and eluting with no retention (Koivikko et al, 2007). On the other hand, Normal phase liquid chromatography (NPLC) was found more convenient to retain polar compounds and better results were observed comparing to RP for the separation of phlorotannins from *F. vesiculosus* (Koivikko et al, 2007).

Steevensz et al (2012) mentioned that to have a better baseline and higher retention time, water content was decreased but peak shape and sensitivity were changed in a negative way. Reasonable results were obtained with a gradient ranging from 5% to 35% aqueous phase. Also adding up to 15% methanol helped to separate peaks but sensitivity and peak shapes were adversely changed. (Steevensz et al, 2012).

Ferreres et al (2012) studied on characterization of most known phlorotannins found in literature such as dioxinodehydroeckol (371), eckol (373), fucophloroethol (375), 7-phloroeckol (497), fucodiphloroethol (499), phlorofucofuroeckol (603), fucotriphloroethol, (623), dieckol (743), and fucophloroethols with six (747), seven (871) and eight units of phloroglucinol (995) using HPLC-DAD-ESI-MS^n.

1.2.3 Lipid oxidation

Lipids provide essential nutrients such as fat-soluble vitamins (A, D, E, and K) and fatty acids such as linoleic and linolenic acids. Lipids have an important role in food products not only because of their nutritional values but also their effect on quality even when they do not constitute a major component in the food. They contribute flavor, odor, texture, color, satiety and palatability of the food (Frankel, 1998).

However, lipids are prone to oxidation which can affect food quality in a negative way. Changes occuring in lipid during oxidation affects not only quality but also influence the nutritional value and safety of food. Lipid oxidation occurring in food products is one of the major concerns in food technology by being responsible for rancid odors and flavors of the products (Rasmy et al., 2012).

There are three types of oxidation mechanisms which can occur in products that include lipids: Autoxidation, enzyme catalysed oxidation and photooxidation.

Because mayonnaise samples were kept in dark and enzymes which can affect the oxidation did not exist in the product, lipid oxidation in mayonnaise samples was considered only autoxidation (Jacobsen, 1999).

1.2.4 Autoxidation mechanism

Lipid autoxidation is a complex process whose mechanism of action has not yet been fully discovered (Shahidi and Zhong, 2011).

Autoxidation, which is also called a free radical chain mechanism, proceeds via three basic steps:

- Initiation
- Propagation
- Termination

Autoxidation also involves initiators and or promoters such as heat, oxygen, transition metals, metalloproteins and/or microorganisms (Shahidi and Zhong, 2011).

Initiation: This is the step where free radicals form:

$$LH \xrightarrow{\text{Initiator (I)}} IH + L\bullet \qquad \textbf{(1.1)}$$

Unsaturated lipids (LH) loose a hydrogen radical (H•) in the presence of initiators such as metal ions, heat, protein radicals to form lipid free radicals (alkyl radicals, L•) (Frankel, 1998).

Propagation: Hydroperoxides occur at this step of autoxidation.

$$L\bullet + O_2 \longrightarrow LOO\bullet \qquad \textbf{(1.2)}$$

$$LOO\bullet + LH \longrightarrow LOOH + L\bullet \qquad \textbf{(1.3)}$$

The alkyl radical of unsaturated lipids (L•) containing a labile hydrogen reacts very rapidly with molecular oxygen to form peroxyl radicals (LOO•) which can react with a new unsaturated fatty acid to form hydroperoxides (LOOH) and a new lipid radical (L•). Lipid radical propagates the chain reactions (Frankel, 1998). Lipid hydroperoxides, which are tasteless and odorless due to their low volatility, are the primary products of autoxidation (Jacobsen and Nielsen, 2007).

The lipid hydroperoxides which are formed in this step, may be decomposed by homolytic β-scission (1.4) to form alkoxyl radicals as showed below (Frankel, 1991).

$$LOOH \longrightarrow LO\bullet + \bullet OH \qquad \textbf{(1.4)}$$

$$LO\bullet + LOOH \longrightarrow LOO\bullet + LOH \qquad \textbf{(1.5)}$$

$$LO\bullet + LH \longrightarrow LOH + L\bullet \qquad \textbf{(1.6)}$$

$$\bullet OH + LH \longrightarrow H_2O + L\bullet \quad \textbf{(1.7)}$$

Both LO• and LOO• radicals can propagate the free radical chain reaction to form new hydroperoxides (Frankel, 1991).

Termination: At this last step, non-radical products occur.

After reaching a maximum, the rate decreases and the peroxyl radicals reach with each other and self-destruct to form non-radical products (LOOL, LOL, L-L) (Frankel, 1998). These non-radical products are also tasteless (Frankel, 1991).

$$LOO\bullet + LOO\bullet \longrightarrow LOOL + O_2 \quad \textbf{(1.8)}$$

$$LOO\bullet + L\bullet \longrightarrow LOOL \quad \textbf{(1.9)}$$

$$L\bullet + L\bullet \longrightarrow L\text{-}L \quad \textbf{(1.10)}$$

$$LO\bullet + LO\bullet \longrightarrow LOOL \quad \textbf{(1.11)}$$

$$LO\bullet + L\bullet \longrightarrow LOL \quad \textbf{(1.12)}$$

In the presence of trace metals such as iron and copper, thermal dissociation and decomposition of hydroperoxides form alkoxyl and peroxyl radical intermaediates (LO• and LOO•) which can propagate the free radical chain reaction (Frankel, 1991).

The primary autoxidation product which is hydroperoxide, can be detected by the so-called Peroxide value (PV) analysis. The Peroxide value can be used as an indicator of the extent of the lipid oxidation.

As EPA and DHA fatty acids contain 5 and 6 double bonds, respectively, they are prone to oxidation (Frankel, 1998). The primary oxidation of EPA and DHA can result in hydroperoxides on the 5-, 8-, 9-, 11-, 12-, 14-, 15-, 18- and 4-, 7-, 8-, 10-, 11-, 13-, 14-16-, 17- 20- carbon, respectively.

The classical mechanism for the free radical oxidation of methyl oleate includes hydrogen abstraction at the allylic carbon-8 and carbon-11 to produce two delocalized three-carbon allylic radicals as shown in the Figure 1.3. According to the mechanism of oleate autoxidation, oxygen attack at the end-carbon positions of these intermediates produces a mixture of four allylic hydroperoxides containing OOH groups on carbons 8, 9, 10 and 11 in equal amounts.

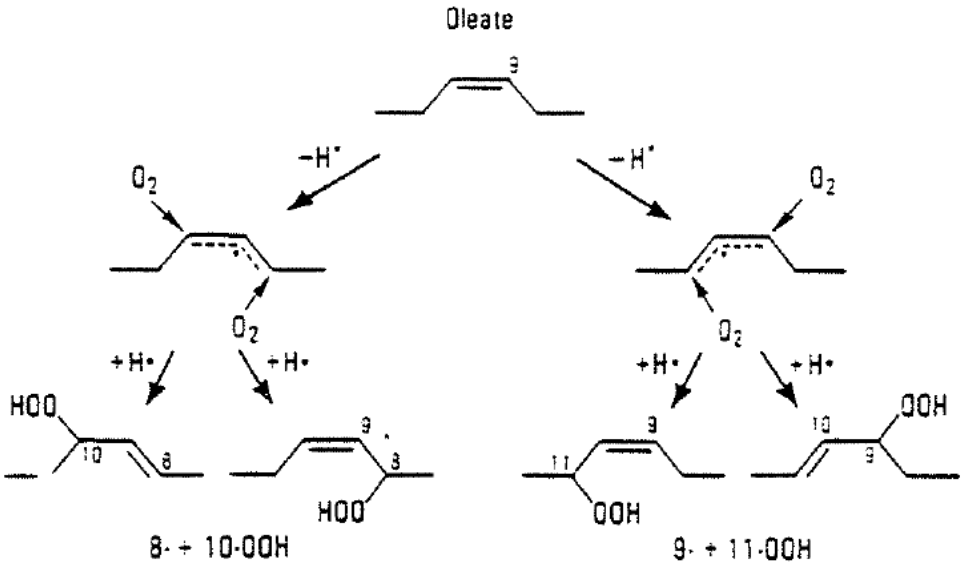

Figure 1.3: The autoxidation of oleate. Adapted from Frankel (1984).

Linoleate is 40 times more active than oleate, as it has an active bis-allylic methylene group on carbon-11 between two double bonds which can lose a hydrogen atom very readily. Hydrogen abstraction at the carbon-11 position of linoleate produces a hybrid pentadienyl radical, which reacts with oxygen at the end carbon-9 and carbon-13 positions to produce a mixture of two conjugated diene 9- and 13-hydroperoxides as hown in the Figure 1.4 (Frankel, 1998).

The reason for the greater activity of linoleate to autoxidation is because of the formation of a pentadienyl radical (Frankel, 1998).

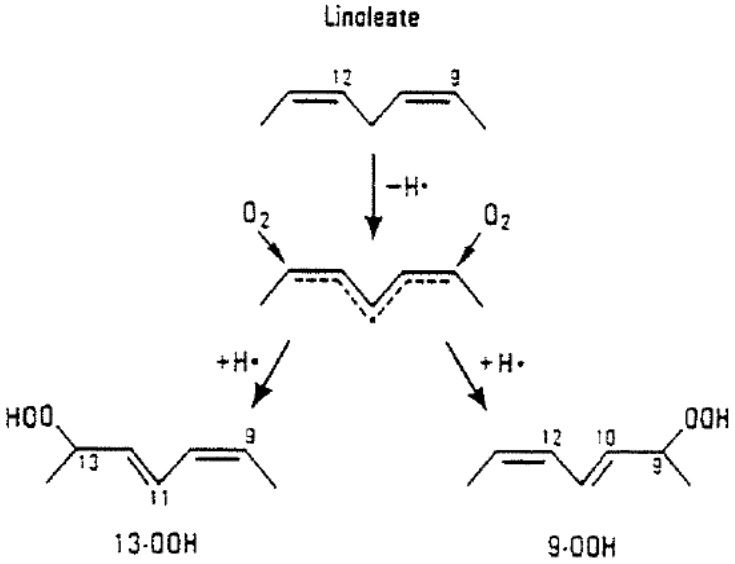

Figure 1.4: The autoxidation of linoleate. Adapted from Frankel (1984).

As methyl linolenate has two bis-allylic methylene groups, it reacts twice as fast with oxygen as linoleate (Figure 1.4). Linoleate was 40 times more reactive than oleate, and linoleate was 2.4 times more active than linoleate (Frankel, 1998).

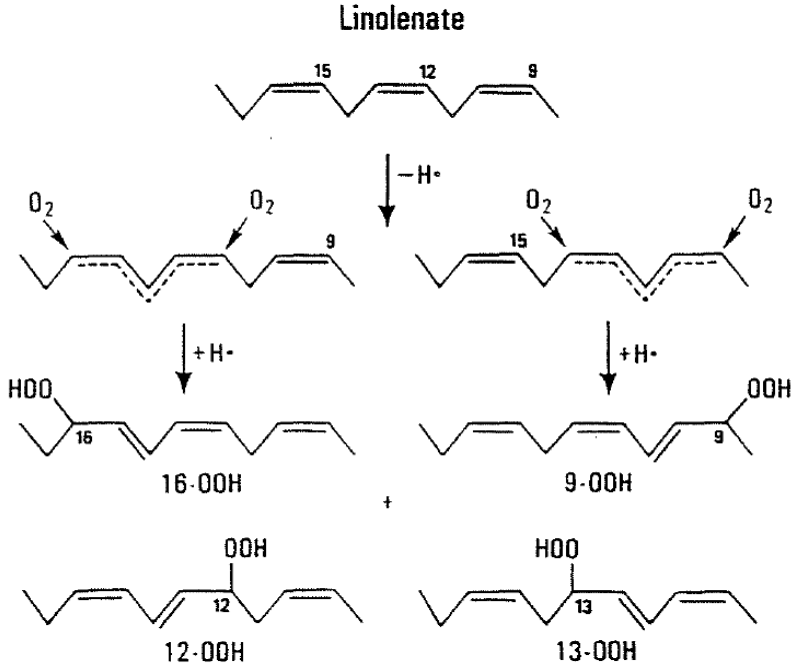

Figure 1.5: The autoxidation of linolenate. Adapted from Frankel (1984).

Hydroperoxides form secondary oxidation products, as they are relatively unstable and can be degraded through different reactions. The decomposition of lipid hydroperoxides produces carbonyl compounds, alcohols and hydrocarbons under

various conditions of elevated temperatures and in the presence of metal catalysts. Homolytic β-scission is a decomposition reaction of hydroperoxides to an intermediary alkoxyl radical, catalyzed by heat or transition metal. Figure 1.6 shows the hemolytic β-scission, the thermal decomposition of monohydroperoxides alkoxyl radicals to form aldehydes, alky and olefinic radicals. The alkyl radicals form either a hydrocarbon by hydrogen abstraction or an alcohol by reacting with a hydroxyl radical. The main volatile decomposition products formed from oleate, linoleate and linolenate are those expected from the cleavage of the alkoxyl radicals (Frankel, 1998).

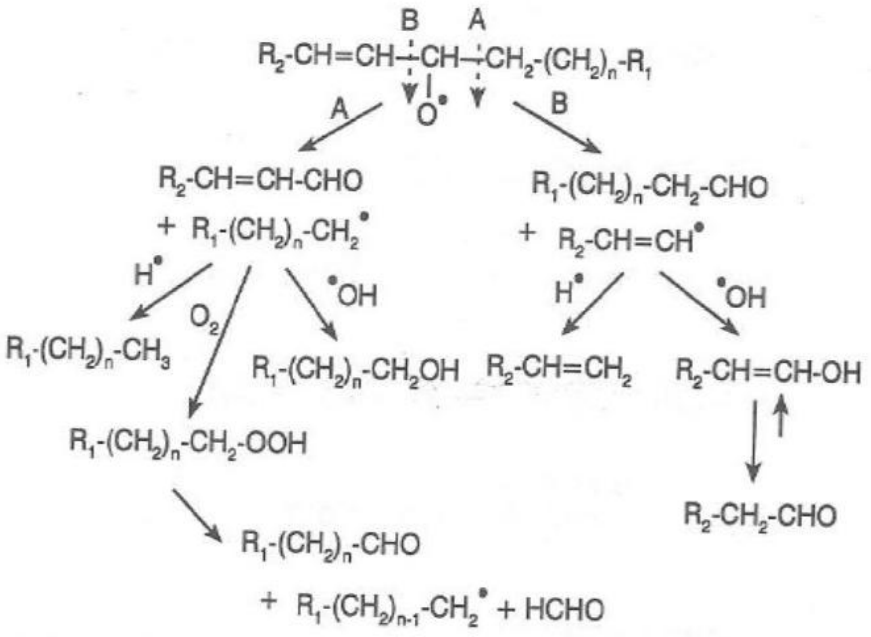

Figure 1.6: The homolytic β-scission of fatty ester monoperoxides. Adapted from Frankel (1998).

Volatile oxidation products, which occur in food after oxidation and give undesirable flavor and odor, are secondary oxidation products. Even the presence of lower than 1 ppm has an impact on food flavor and odor (Frankel, 1998). The volatile oxidation compounds can affect the taste and odor in different ways. Rancid, fishy, metallic and green are some of the words often used to describe the odor and flavor of volatile compounds (Frankel, 1998; Lee et al., 2003).

1.2.5 Antioxidant mechanism

Antioxidants prevent the reaction of free radicals and protect the nutritional values and physiological properties of food products which are prone to oxidation. Due to

the demand of changing synthetic antioxidants with natural antioxidants, effects of several plant-based sources such as spices and herbs as well as seaweeds have been researched.

Antioxidants can work in two mechanisms which are primary antioxidants and secondary antioxidants. Primary antioxidants are called as free radical scavengers or chain-breaking mechanism. Primary antioxidant donates an electron to the free radical present in the system and reacts with the alkyl radicals. Thus, formation of hydroperoxides is stopped and the chain reaction is inhibited (Jacobsen and Nielsen, 2007). Secondary antioxidants act by a number of different mechanisms including metal chelation, oxygen scavenging, and replenishing hydrogen to primary antioxidants. They can work through two mechanisms which are inhibiting the prooxidants and regeneration of the primary antioxidants. Prooxidant inhibition can be done by metal chelation and oxygen scavenging. Regeneration of the primary antioxidant prolongs the effects of primary antioxidant. Additionally, secondary antioxidants often show synergistic effects with primary antioxidants (Frankel, 1998).

Antioxidants can prevent or delay oxidation by scavenging free radicals, quenching singlet oxygen, inactivating peroxides and other reactive oxygen species (ROS), chelating pro-oxidant metal ions, quenching secondary oxidation products, and inhibiting pro-oxidative enzymes (Shahidi and Zhong, 2011).

Chemical structures of the antioxidants have an important impact on its effectiveness. Other factors which may influence the effectiveness of the antioxidants are concentration, temperature, type of oxidation substrate, physical state of the system media, and the presence of the antagonists and synergists (Yanishlieva-Maslarova, 2001). All these factors should be taken into consideration when designing the antioxidant application (Shahidi and zhong, 2011).

It is known that antioxidants behave differently when used in bulk oil and oil-in-water emulsion systems (Jacobsen, 1999; Shahidi and Zhong, 2011). The ability of antioxidants to inhibit lipid oxidation in food emulsions depends on factors such as antioxidant concentration; reactivity; partitioning between oil, water and interfacial phases; interactions with other food components; and environmental conditions such as pH, ionic strength and temperature (Frankel, 1998).

1.2.6 Bioavailability

Besides the investigation of antioxidant potential of phlorotannin extracts in food emulsion systems, it is also of critical importance to evaluate the composition and concentration of antioxidants that are available in the human gastrointestinal system. Bioavailability is closely related to susceptibility to conjugation when the components are incorporated through the intestines, because most of the activity is contributed by free forms and conjugation usually makes it difficult to exhibit activity (Shimizu *et al.*, 2004). Absorption, metabolism, tissue and organ distribution, and excretion data synthesis should be concerned while assessing true bioavailability of any class of phytochemicals.

Studies carried out *in-vivo* systems are complex, expensive and time consuming. On the other hand, *in vitro* studies provide to analyze multiple samples and may show data about relative potential bioavailability of different polyphenolic components (McDougall *et al.*, 2005). One of the objectives of this study was to investigate *in vitro* bioavailability of phlorotannins extracted from *F. vesiculosus*.

1.2.7 Food emulsions

Food emulsions were grouped as milk products such as milk, cream, butter, ice cream, yogurt, cheese; beverage emulsions such as tea, coffee, milk, infant formula, sports drinks, fruit drinks, and colas; dressings such as mayonnaise, salad, French, Italian, Russian, Blue Cheese, Ranch, and Thousand island dressings (McClements, 2005). Mayonnaise is a good representative for a complex food emulsion.

1.2.7.2 Mayonnaise

Mayonnaise is an oil-in-water emulsion which consists of oil droplets dispersed in aqueous continuous phase. Emulsions consist a continuous phase, interfacial region and interior of the droplets which are water, emulsifiers and other surface active compounds, and oil for oil-in-water emulsions, respectively (McClements and Decker, 2000; Shahidi and Zhong, 2011).

Mayonnaise as a food product includes high amount of oil (70-80%) and a low pH (around 4) compared to other food systems (Jacobsen, 2010).

Mayonnaise enriched with fish oil only (70%) and without antioxidants has a poor oxidative stability with a one day shelf life at room temperature (Jafar et al., 1994). It is possible to extent the shelf life of the mayonnaise by adding antioxidants such as

citric acid or sodium citrate and propyl gallate in the oil phase or EDTA and ascorbic acid in the aqueous phase (Jafar et al, 1994). Due to the fact that it is difficult to keep the end food product stable towards oxidation by the addition of only fish oil, using fish oil as a substitution with some part of other vegetable oils were preferred (Jacobsen, 2010). At the present study 20% of rape seed oil was substituted with fish oil and oil content was 80% of total mayonnaise.

Factors which can influence the oxidative stability of the fish oil enriched oil-in water emulsions

The mechanism for lipid oxidation in oil-in-water emulsions differ from bulk lipids because emulsions have an aqueous phase which contains both prooxidants and antioxidants as well as oil-water interface which can effect interactions between oil and water components (Waraho et al, 2011). As our food product is fish oil enriched mayonnaise, discussion will be focused on this particular food product.

Effect of ingredients

Oil-in-water emulsions include many ingredients which may act as either prooxidants or antioxidants, or they may not influence the oxidation rate at all (Jacobsen, 1999). In mayonnaise, oil, water, sugar, salt, proteins and amino acids, egg yolk, air and oxygen, transition metals have effects on oxidative stability of the product.

Oil

Mayonnaise enriched with fish oil includes high amounts of unsaturated fatty acids which directly affects the oxidation stability. Oxidation rate of fatty acids increases with the increasing degree of unsaturation (Frankel, 1998). Increasing the fish oil amount in the mayonnaise increases the oxidation level of mayonnaise (Jacobsen, 1999). However, oxidation also depends on the other ingredients' impacts, because of this reason it is hard to predict (Jacobsen, 1999).

Water

Lipid oxidation rate is affected by the water in foods due to metal ions can solve in water and also contain transition metals itself. On the other hand, water may decrease the lipid oxidation by the formation of hydrogen bonds between water and lipid hydroperoxides (Jacobsen, 1999).

Sugar

Carbohydrates in high concentrations are capable of scavenging free radicals and they may decrease oxidation in emulsions by decreasing the oxygen concentration in

the aqueous phase (Coupland and McClements, 1996). This decrease may have an impact on the propagation step of autoxidaton. Addition of sugar increases the viscosity and it is reported that viscous food products are more stable than the less viscous ones (Reinelt et al, 1979).

Salt

Salt may have prooxidative or antioxidative effect according to its concentration in the food system. Another effect of salt on oxidation is linked with the charge of the emulsifier (Mei et al, 1998). The effect of salt on oxidation also depended on the charge of the ferrous iron concentration (Jacobsen, 1999).

Proteins and aminoacids

Proteins and aminoacids have been reported to have prooxidant and antioxidant effects in oil-in-water emulsions (Coupland and McClements, 1996).

Egg yolk

Egg yolk does not have a promoting effect on oxidation in mayonnaise, on the contrary it may have antioxidant effect with its phosvitin and LDL content depending on the pH of the emulsion system (Jacobsen, 1999).

Air and oxygen

Oxidation occurs in the presence of oxygen. Therefore oxidation increases with the increasing amount of oxygen in the headspace of the emulsion (Jacobsen, 1999).

pH and emulsifier

According to Jacobsen et al. (2001a) a decrease in pH value from neutral to around four in fish oil enriched mayonnaise, iron bridges between phosvitin, low density lipoproteins and lipovitellins which causes the release of iron from the egg yolk. These released irons can increase lipid oxidation in fish oil enriched mayonnaise. Another study by Horn et al. (2011) was reported that emulsions prepared with proteins at pH 7 oxidized less than the ones prepared at pH 4.5.

Sørensen et al. (2010a) were hypotesized that substituting egg yolk with a less iron-containing emulsifier (milk protein based emulsifier was used) might increase the oxidative stability of fish enriched mayonnaises, however, the PV level for milk protein based emulsifier was around 100-fold higher than the PV level in egg yolk. There fore, initial quality of the emulsifiers seemed to be more important than their content of iron.

Effect of droplet size

Mean radius of the droplets in mayonnaise is around 1-2 μm and distribution of droplet sizes is ranging from 0.1 to over 10 μm (McClements, 2005).

Average thickness of the interfacial membrane, which is a layer between oil droplet and aqueous phase, is around 14 μm and this layer includes the components such as surface active proteins and lecithin-protein granules from egg yolk (Langton et al., 1999; Ford et al., 2004).

Even though emulsion droplets in food can vary from 0.2 μm to 100 μm, droplet size does not change lipid oxidation rates conspicuously, so it has a small impact on lipid oxidation rate (McClements and Decker, 2000; Horn et al., 2011). The mean droplet diameter in food emulsions ranges from less than 100 nm to greater than 100 μm (McClements et al., 2007).

According to Jacobsen et al. (2000), droplet size of fish oil enriched mayonnaises influenced the lipid oxidation. Results were observed that mayonnaise samples with smaller droplet sizes were oxidised faster at the initial part of the storage period than the ones with larger droplet sizes. However, at the later part of the storage experiment, no influence of droplet size was observed on oxidative flavour. This was explained by the effect of large interfacial area of the small droplets that increased the contact area between iron and lipid hydroperoxides which were located in the aqueous phase and interface, respectively, and oxidation rate was increased comparing to larger droplets. At the later stages of storage, oxidation started to occur more into the oil droplets. Thus, droplet size becomes less important in affecting the oxidation rate.

1.2.8 Lipid oxidation and antioxidants in emulsions

The rate of lipid oxidation in oil-in-water emulsions can be effected by many factors such as fatty acid composition, pH of aquous phase, ionic composition, type and concentration of antioxidants and prooxidants, oxygen concentration, lipid droplet characteristics (particle size, concentration and physical state and emulsion droplet interfacial properties such as thickness, charge, rheology, and permeability (McClements and Decker, 2000).

Due to the fact that emulsions have both aqueous and oil phase, their lipid oxidation mechanism is different than the bulk lipids. There is a theory called polar paradox which explains the behaviour of antioxidant in different systems based on polarity.

On the other side, another group of hypothesis called cut-off effect with a different approach comparing to polar paradox theory.

1.2.9 Polar paradox theory and cut-off effect

Polar Paradox Theory proposes that polar antioxidants are more effective in less polar media such as bulk oils and nonpolar antioxidants are more effective in relatively more polar media such as oil-in-water emulsions (Shahidi and Zhong, 2011). This hypothesis has been tested and confirmed by many studies which applied antioxidants with different polarities. Although it is accepted, recently some of the studies disagree with the polar paradox theory (Shahidi and Zhong, 2011).

Studies that are practised with bulk oils display different results compared to the emulsion food systems due to the fact that surface volume ratios are different which can effect the oxidation mechanism and antioxidant behavior; bulk oils have low surface/volume ratio and emulsions have high surface/volume ratio (Shahidi and Zhong, 2011).

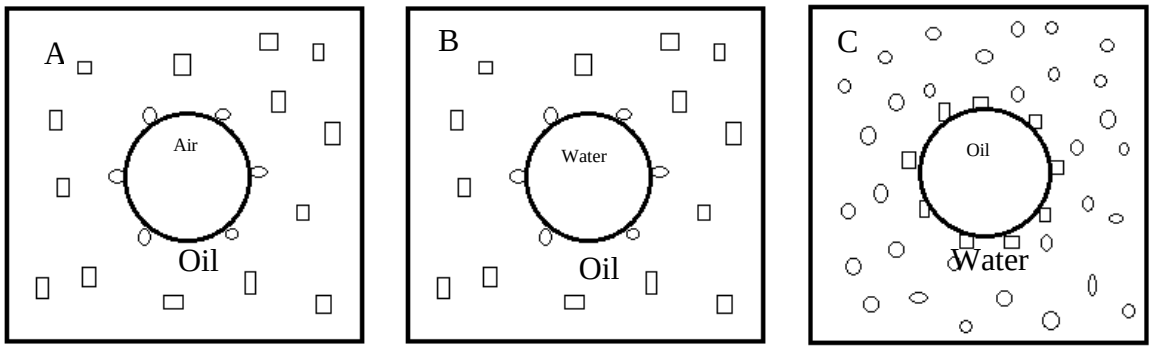

Figure 1.7: Distribution of antioxidants in (A and B) bulk oil and (C) oil-in-water emulsion according to interfacial phenomena and polar paradox (□:Hydrophobic antioxidant,O : Hydrophilic antioxidant) (Adapted from Shahidi and Zhong, 2011).

In bulk oils (Figure 1.7A), the oil-air interface was considered to be the site where oxidation was initiated and propagated to the inner part of oil. The partially fat-soluble polar antioxidants were placed in the oil-air interface where surface oxidation occurs. Due to the fact that oil is more polar than air, this distribution is questioned (Shadidi and Zhong, 2011). As shown in Figure 1.7B, polar antioxidants are located at the interface of the oil-water interface and inhibit oxidation more effectively than

nonpolar ones, instead of being located at the oil-air interface which was believed previously (Shahidi and Zhong, 2011).

Most of the food lipids naturally exist in the form of emulsions and it is well known that emulsions are more susceptible to oxidation than bulk oils. An oil-in-water emulsion consists of three main parts which are lipid droplets, continuous water phase and interfacial area which is between oil and water and where emulsifiers and other surface active components take place (Shahidi and Zhong, 2011). According to polar paradox, oil-in-water emulsions are better protected from oxidation by nonpolar antioxidants than by polar ones, in contrast to bulk oils. This is because nonpolar or amphiphilic antioxidants are gathered at the oil-water interface and formed a protective membrane around lipid droplet, while polar antioxidants are distributed in the aqueous phase (Figure 1.7C) (Frankel, 1994; 2001).

However, recently, some studies have emerged new evidences which contradicts the polar paradox theory, therefore, more complex factors in addition to polarity must be taken into account to explain antioxidant efficacy. Additionally, some results showing that not all antioxidants behave in the same manner proposed by polar paradox. Antioxidant activity of phenolic compounds in oil-in-water emulsions is affected by physicochemical phenomena such as partitioning in the different phases, diffusion and self-aggregation and association of antioxidants with amphiphiles, which are, in turn mainly influenced by hydrophobicity (Laguerre et al., 2009; 2013). Laguerre et al. (2013) concluded that the polar paradox hypothesis was too simple to predict antioxidant efficacy in a complex food emulsion system which is catalyzed by iron stemming from the egg yolk used as an emulsifier. Thus, cut-off effect, which contradicts the polar paradox, showed that the antioxidant capacity increased as the alkyl chain was lengthened until a threshold in hydrophobicity was reached (Laguerre et al., 2009). Beyond this threshold, the antioxidant capacity suddenly collapsed. This cut-off effect was confirmed by some studies which were done using rosmarinate alkyl esters, chlorogenate alkyl esters, hydroxytyrosol fatty esters and found that antioxidant capacity of these compounds was increasing as the alkyl chain was lengthened until a critical point, then decreasing with the increasing alkyl chain length (Laguerre et al., 2009, Medina et al., 2009, Laguerre et al., 2010).

Laguerre et al. (2012) suggested three putative mechanisms behind the cut-off effect in order to explain the cut-off phenomenon; the “reduced mobility”, the “internalization” and the “self-aggregation” hypothesis. As explained by Laguerre et

al. (2013), the reduced mobility hypothesis suggested that the mobility of antioxidant decreases with the increasing alkyl chain length. The internalization hypothesis suggested that increasing the hydrocarbon chain from medium to long chains could move the antioxidant away from the interface into the lipid core of emulsion where an antioxidant would be a poor antioxidant. The self-aggregation hypothesis proposed that beyond the critical chain length the antioxidant capacity collapse occurs because of antioxidant self-aggregation and the fact that long-chain antioxidants mainly exist as colloidal aggregates. Self-aggregation could have two drawbacks, first, removing the antioxidant from the interface where oxidation is most prevalent. Second, micellization makes long-chain antioxidants bulkier than free molecules, which obviously makes them less mobile toward the oxidizable substrate, free radicals, and transition metals.

2. EXPERIMENTAL DESIGN

The aim of this thesis was to study the antioxidative effect of *F. vesiculosus* extracts' oxidative stability in fish oil enriched mayonnaise. Water and ethylacetate solvents, which are considered as a food-grade solvent, were used in order to extract the phlorotannins from *F. vesiculosus* and determined which concentration of these extracts has a better effect on preventing the fish oil enriched mayonnaise system from lipid oxidation. Different concentrations of *F. vesiculosus* crude water and ethylacetate extracts (0.5, 1, 1.5, 2 mg/mL) were tested. Antioxidant activity of the water and ethylacetate extracts were determined using three different assays which were DPPH scavenging activity, reducing power and metal chelating activity.

After the results were evaluted, 1000, 1500 and 2000 μg/mL concentrations were chosen to add into the mayonnaise as shown in the

Table 2.1, due to the fact that concentration of 500 μg/mL showed relatively lower reducing power and metal chelating activity comparing to the others. One mayonnaise sample was produced as a reference without addition of extract, three samples were produced with three different concentrations (1, 1.5 and 2 g/kg) of water extracts of *F. vesiculosus* and three samples were produced with the same concentrations (1, 1.5 and 2 g/kg) of ethylacetate extracts of *F. vesiculosus.* Thus, the effects on oxidative progress of adding different types and different amounts of these extracts were aimed to be observed.

Table 2.1: Added concentrations of phlorotannin extracts into the mayonnaise.

Ingredients	**Amount (g) in 1000 g mayonnaise**						
	Mayo_ REF	Mayo_ WE1	Mayo_ WE2	Mayo_ WE3	Mayo_ EAE1	Mayo_ EAE2	Mayo_ EAE3
Mayonnaise	1000	1000	1000	1000	1000	1000	1000
WE	-	1	1.5	2	-	-	-
EAE	-	-	-	-	1	1.5	2

The study was tested with 1, 1.5 and 2 g/kg ethylacetate and water extracts of *F. vesiculosus* in 16% fish oil enriched mayonnaise. Fish oil was 20% of the total oil content, this ratio was chosen according to the studies done by Jacobsen (1995) and Meyer and Jacobsen, (1996). The oxidative stability of mayonnaises enriched with fish oil was studied through a storage experiment. During the storage experiment samples were taken for the analysis which can show the oxidation progress. Mayonnaise samples were kept at 20 °C in the dark. The production of all mayonnaise samples were made at the same day in order to be sure that oxidative stage of the samples was similar at the beginning.

3. MATERIALS AND METHODS

3.1 Materials

Mayonnaise consists of rape seed oil, fish oil, potassium sorbate – E202, egg powder, yellow color, sugar, salt, xanthan gum FG – E415, lemon juice concentrate 48-Brix, lactic acid 60% -E270, acetic acid 20% and distilled water. As an antioxidant, water and ethylacetate extracts of *F. vesiculosus* were used. Other materials used in the study are as follows:

- Rape seed oil: The rape seed oil used was a refined oil produced by Maritex 22/8-05. Rape seed oil was kept at -40°C in the freezer until used.
- Fish oil: The fish oil used was a refined non-deodorized fish oil without added antioxidants, Maritex no 43-01. The fish oil was produced by Maritex A/S (Sortland, Norway). The fish oil was stored at -40°C until used.
- *F. vesiculosus* extracts: Water and ethylacetate extracts of *F. vesiculosus* (Linnaeus) were provided by Matís ohf., Vínlandsleid 12, 113 Reykjavík, Iceland.
- Potassium sorbate – E202: : Merck, Darmstadt, Germany
- Whole egg powder: Sanovo Foods, Odense, Denmark
- Yellow color – E160a: Unknown producer, received from mayonnaise producer Crown Foods, Mørkøv, Denmark
- Sugar: Dansukker from Nordic Sugar, Copenhagen, Denmark
- Salt: Local supermarket
- Xanthan gum FG – E415: Unknown producer, received from mayonnaise producer Crown Food, Mørkøv, Denmark
- Lemon juice concentrate 48-Brix: Unknown producer, received from mayonnaise producer Crown Food, Denmark
- Lactic acid 60% -E270: Sigma, Germany.
- Acetic acid 20%: Sigma
- Distilled water: DTU orange colored tap.

3.2 Methods

3.2.1 Extraction of phlorotannins from *F. vesiculosus*

Brown algae *F. vesiculosus* was extracted by Wang et al (2012) and sent to our laboratory for the present study. In a few words, 2 gr of dried algal powder was dissolved in 50 mL of ethanol/water (80:20, v/v) and incubated in a platform shaker for 24 h at 200 rpm and at room temperature. The mixture was cenrifugated at 2500 *g* for 10 min at 4 °C and filtered with Whatman no. 4 filter paper.

The crude 80% ethanol extract was subjected to solvent fractionation. The dried algal powder (40 g) was extracted with 200 mL of 80% ethanol by the same procedure described above.

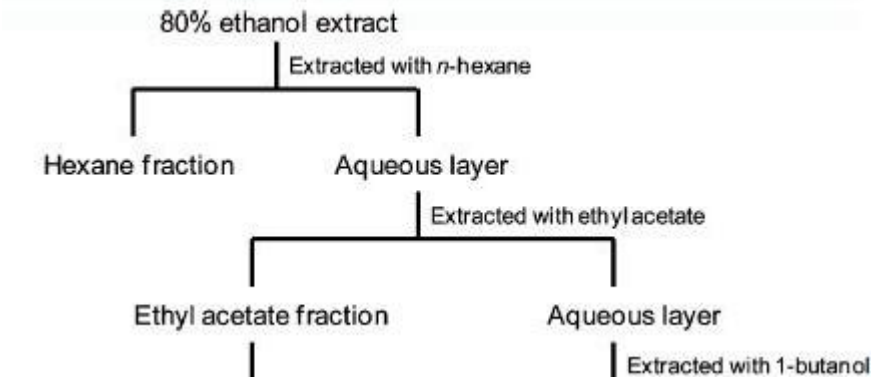

Figure 3.1: Flow chart of extraction and fractionation of *F. vesiculosus* (Wang et al, 2012).

The extract was concentrated in vacuum to a small volume, and the residue was suspended in a mixture of methanol and water (40:30, v/v) and partitioned three times with n-hexane, ethyl acetate, and 1-butanol successively (Figure 3.1). After the solvent was removed and freeze dried, four fractions were obtained, the n-hexane, ethyl acetate, and 1-butanol soluble fractions and an aqueous residue.

3.2.2 Fractionation of ethylacetate extracts of *F. vesiculosus*

1 g of crude extract (ethylacetate and water crude extracts) is weighed in a centrifuge glasses. 10 ml of solvent (1=ethylacetate, 2=heptanes, 3=methanol and 4=dichloromethane) is added on the crude extract and mixed. Mixture is incubated at 150 rpm for an hour in shaker and centrifugated at 1290 *g* for 10 min at 4°C. Supernatant is removed to another tube and 10 ml of solvent is added. This process is

repeated 2 times more. Every extraction was in duplicate and named “Purified extracts”.

Ethylacetate extracts of Purified Ethylacetate Extracts (PEAE) were used for further purification on Oasis MAX (Reversed Phase), Amino (Normal Phase) and Diol (Normal Phase) SPE cartridges.

The Oasis MAX cartridge was preconditioned with 2 times column volume (6.5 ml) of 100% methanol and followed by 1 column volume of 100% water (2% ammonium hydroxide pH 11). Sample solution was prepared by diluting 10 ml of PEAE-EA1 solution in 10 ml water (2% ammonium hydroxide). Sample was loaded and column was washed with 1 column volume of 25% methanol (pH 7) to collect fraction 1. To obtain fraction 2, compounds were eluted with 1 column volume of 60% methanol (pH 7); for fraction 3, compounds eluted with 1 column volume of 100% methanol (pH 7). For fraction 4, half column volume of 100% methanol (pH 7) and half column volume of 25% methanol (1%formic acid, pH 2) were eluted. For fraction 5, 1 column volume of 60% methanol (1% formic acid, pH 2) was eluted. Fraction 6 was obtained by eluting 1 column volume of 100% methanol (1% formic acid, pH 2). Then 100 µL of each fraction were taken for LC-MS analysis.

The Amino and Diol cartridges were preconditioned with 1 column volume of (6.5 ml) 50% dichloromethane. Sample solution was prepared by diluting PEAE-EA2 with ethylacetate:dichloromethane (1:1). Sample was loaded and column was washed with 1 column volume of dichloromethane:ethylacetate (1:1) (pH 7) to obtain fraction 1. To collect fraction 2, compounds were eluted with 1 column volume of 25% dichloromethane in ethylacetate (pH 7). For fraction 3, compounds eluted with 1 column volume of 100% ethylacetate (pH 7). For fraction 4, compounds eluted with 1 column volume of 75% ethylacetate in methanol (pH 7). Fraction 5 was obtained by using 1 column volume of 100% methanol (pH 7). All fractions were evaporated under nitrogen gas. Amino fractions were redissolved in 2 ml of 75% methanol. Diol fractions were redissolved in 1ml 50% methanol. For LC-MS analysis, 50 µl of each fraction were taken in to vials.

3.2.3 Characterization of *F. vesiculosus* purified extracts and fractions by HPLC/DAD-MS

Characterization of *F. vesiculosus* purified ethylacetate extract and its fractions were analysed using HPLC/DAD-MS (Agilent Technologies). Mobile phase was 2mM formic acid (buffer, pH 3.3). Gradient was 100% w (almost 100% acetonitrile). As a stationary phase phenyl column was used and injection volume was 0.2 µL. qTOF was used in both positive and negative ionization mode, full scan was selected between 50-1600 m/z. 8 scans were done in one second.

3.2.4 Total phlorotannin content

Total phlorotannin content of *F. vesiculosus* extracts were determined according to the method of Wang et al. (2012). Ethylacetate and water extracts of *F. vesiculosus* samples were dissolved in methanol. Sample solutions were prepared in different concentrations (0.1, 0.2, 0.4, 0.8, 1, 2, 4, 8 mg/mL). One milliliter aliquot of sample solution was mixed with 5 mL of Folin−Ciocalteu reagent (10% in distilled water). After 5 min, 4 mL of sodium carbonate (7.5% in distilled water) was added. The samples were incubated for 2 h at room temperature in the dark. The absorbance was measured at 725 nm with a UV−vis spectrophotometer (Shimadzu UV mini 1240, Duisburg, Germany). A standard curve with serial phloroglucinol solutions was used for calibration. Five different concentration points of phloroglucinol and a blank were prepared vary from 0 to 100 µg/mL, the absorbances of these six points were measured by the spectrophotometer, after that, the standard had been plot. Calculation was done by plotting a phloroglucinol standard curve (R^2=0.999). The results are expressed as grams of phloroglucinol equivalents (PGEs) per 100 g of extract.

3.2.5 Antioxidant activity assays

In order to determine the antioxidant activity of *F. vesiculosus* crude extracts; DPPH scavenging activity, ferrous ion chelating and reducing power assays were applied.

3.2.5.1 DPPH radical scavenging activity

The antioxidant activity of samples was determined by the method described by Yang et al. (2008). Samples and butylated hydroxyl toluene (BHT) were prepared at different concentrations (0.5, 1.0, 1.5, 2.0 mg/mL) in methanol. 150 µL of samples were added into the eppendorf tubes and 150 µL of 0.1 mM DPPH was added to

each eppendorf tubes and vortexed. 200 µL of the mixture was added to the microtiterplate and read after 30 min using a BioTek Synergy 2 Multi-Detection Microplate Readers.

3.2.5.2 Ferreus ion chelating

Fe^{2+} ion chelating activity of the phlorotannin extracts (WE and EAE) was determined according to Farvin et al. (2010). Samples and ethylenediaminetetraacetic acid (EDTA) were prepared at different concentrations (0.5, 1.0, 1.5, 2.0 mg/mL) in methanol. 200 µL of sample was put in an eppendorf tube and 270 µL distilled water was added. To start the reactions, 10 µL 2mM ferrous chloride was added and vortexed. After 3 min, 20 µL 5 mM ferrozine was added and vortexed. 250 µL of the mixture were taken to the microtiterplate and read after 10 min at 562 nm using a BioTek Synergy 2 Multi-Detection Microplate Readers. The chelating capacity was calculated as follows.

Iron chelating activity (%) = [1-((Abs. of sample – Abs. of sample control)/ Abs. of Blank)] x100

3.2.5.3 Reducing Power

The reducing power of phlorotannin extracts was determined according to the method of Yang et al. (2008). Samples and ascorbic acid (AA) were prepared at different concentrations (0.5, 1.0, 1.5, 2.0 mg/mL) in methanol. 0.2 mL of the sample was transferred to eppendorf tubes. 0.2 mL of 0.2 M phosphate buffer and 0.2 mL of 1% potassium ferricyanide were added. Samples were incubated at 50 °C for 20 min. After incubation, 0.2 mL of 10% TCA was added and sample was mixed. 228 µL of this reaction mixture was transferred to a new eppendorf tube and mixed with 228 µL of distilled water. 46 µL of 0.1% ferric chloride was added to the sample and incubated for 10 min at room temperature. 200 µL of each sample were put into the microtiterplate and read at 700 nm using a BioTek Synergy 2 Multi-Detection Microplate Readers.

3.2.6 Determination of bioavailability by in vitro gastrointestinal digestion method

In vitro gastrointestinal digestion system method was performed according to the procedure described by McDougall *et al*. (2005). Release of phytochemicals from *F. vesiculosus* extracts was analyzed at different stages of digestion. These stages

represented the aliquots from gastric digesta (post gastric, PG) and from GI (gastro-intestinal) digesta, including IN (representing the material that entered the serum; dialyzable fraction) and OUT (representing the material that remained in the GI tract, undialyzable fraction). PG, IN and OUT samples were stored at -20ºC until further analysis. These were thawed and centrifuged at 23000 *g* prior to analysis. Total phlorotannin content and antioxidant capacity were determined for each of these PG, IN and OUT samples using the methods described above.

As shown in the Figure 3.2, 0.5 g of extract was weighed and mixed with 20 mL distilled water and mixed till homogenised in a glass beaker. After that, 1,5 mL pepsin was added and pH was adjusted to 1.7 using 5 N HCl in order to mimic the gastric conditions. Beaker was covered with parafilm and placed in a water bath for 2 hours at 37°C and 100 rpm. After 2 hours, 2 mL sample was taken and kept at -20°C for further analysis as a post gastric (PG) fraction. After taking sample, 4.5 mL pancreatin and 4.5 mL bile salt were added into the beaker. After that, pre-prepared dialysis tube (Sigma-Aldrich, D9652-100FT dialysis tubing cellulose membrane, avg. Flat width 33 mm, USA), which was cut 15 cm long, was knotted on one side and 20 mL of $NaHCO_3$ was pipetted into the dialysis tube and the other side was also knotted and then placed into the beaker. To mimic the small intestine conditions, beakers were put into the water bath again for 2 hours at 37°C and 100 rpm. After 2 hours, samples were taken from inside the dialysis tube which was called “IN” fraction and outside of the dialysis tube (from the beaker) which was called “OUT” fraction. IN and OUT fractions were also kept in -20°C for the further analysis. Before the analysis, PG, IN and OUT fractions were centrifuged at 1800 rpm for 5 minutes and supernatant was used for the antioxidant activity and total phlorotannin content analysis.

Sample Preparation

•0,5 g of sample + 20 mL of distilled water
•Mix them untill homogenized in a beaker
•pepsin is added and pH is adjusted to 1,7 with HCl

Gastric Phase

- Pepsin/ HCl digestion in a water bath for 2 h at 37°C
- PG(Post Gastric) fractions

Small Intestinal Phase

•Na2CO3 solution is ejected into dialysis tube and put it into the beaker after gastric phase
•Bile salts/pancreatin are added for 2 h at 37 °C to simulate small intestine.
•IN and OUT samples are taken from inside and outsıde of the dialysis tube

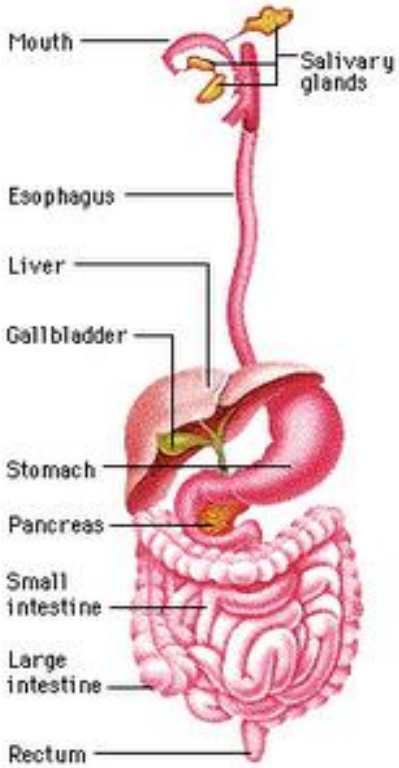

Figure 3.2: *In vitro* gastrointestinal digestion system.

3.2.7 Production of mayonnaise

The aim is to evaluate the oxidative stability of mayonnaise enriched with 16% fish oil (64% rape seed oil) when different concentrations of two types of crude Icelandic *F. vesiculosus* extracts (water, WE, and ethyl acetate, EAE) is added. The WE and EAE are chosen based on antioxidant screening by *in vitro* antioxidant assays. Sample codes were mentioned in the Table 3.1.

Table 3.1: Produced mayonnaise samples and their code names.

Sample	Code
Mayonnaise without antioxidant (reference, neg. control)	Mayo_REF
Mayonnaise with WE conc. 1	Mayo_WE1
Mayonnaise with WE conc. 2	Mayo_WE2
Mayonnaise with WE conc. 3	Mayo_WE3
Mayonnaise with EAE conc. 1	Mayo_EAE1
Mayonnaise with EAE conc. 2	Mayo_EAE2
Mayonnaise with EAE conc. 3	Mayo_EAE3

The dried extracts were dissolved in the water phase during the mayonnaise production. Concentrations of extracts were 0, 1, 1.5 and 2 g dried extract/1 kg mayonnaise (Table 3.2).

Table 3.2: Added concentrations of phlorotannin extracts into the mayonnaise.

Ingredients	**Amount (g) in 1000 g mayonnaise**						
	Mayo_ REF	Mayo_ WE1	Mayo_ WE2	Mayo_ WE3	Mayo_ EAE1	Mayo_ EAE2	Mayo_ EAE3
Mayonnaise	1000	1000	1000	1000	1000	1000	1000
WE	-	1	1,5	2	-	-	-
EAE	-	-	-	-	1	1,5	2

Ingredients (per kg mayonnaise):

Water (182.45 g), potassium sorbate E202 (0.80g), A heat-stable egg powder (20.00 g), yellow color liquid E160a (0.05 g), sugar (13.00), Salt fine raff. (6.00 g) Rape seed oil (614.80 g), fish oil (153.70 g), FG E415 Xanthan gum (0.50 g), lemon concentrate 48 Brix (0.90 g), 80% lactic acid E270 (0.60 g), acetic acid 20% (7.20 g) (Table 3.3).

Table 3.3: The mayonnaise was enriched with fish oil (20% of rape seed oil is replaced with fish oil).

Ingredient	**Amount [g]**
Water	182.45
Potassium sorbate – E202	0.80
Egg powder	20.00
Yellow color, – E160a	0.05
----------	----------
Sugar	13.00
Salt	6.00
----------	----------
Rape seed oil	614.80
Fish oil	153.70
----------	----------
Xanthan gum FG – E415	0.50
----------	----------
Lemon juice concentrate 48-Brix	0.90
Lactic acid 80% - E270	0.60
Acetic acid, 20%	7.20

Production method of the mayonnaise:

1. All ingredients were weighed
2. Water and liquid yellow color (E160a) were mixed
3. Potassium sorbate (E202), and egg yolk powder was dissolved in the mixture of water and the yellow color
4. Salt and sugar were then dissolved in the same mixture. Phlorotannin extracts were added into the mixture. This mixture was poured into the Stephan mixer and mixed for 15 seconds under vacuum.

5. Oil (a small amount) and Xanthan gum were mixed and added the mixture in Stephan mixer. Then mixture was blended in 15 seconds again under vacuum.
6. The large portion of oil was mixed with lemon concentrate, lactic acid and acetic acid
7. The oil mixture is slowly poured into the Stephan mixer using a funnel, while mixed without vacuum for 2 minutes.
8. Finally, whole mixture was mixed under vacuum for 30 seconds.

Mayonnaise production was performed on 3rd of April and storage experiment was started at the same day. Mayonnaise recipe was applied as it is described above. Mayonnaise mixed with the concentration of 2g/kg ethylacetate extract of *F. vesiculosus* did not become a mayonnaise form.

And while producing mayonnaise mixed with the concentration of 2 g/kg water extract of *F. vesiculosus*, salt and sugar were added at the end of the mayonnaise production instead of mixing them with the water phase as was done for the others.

3.2.8 Storage conditions and sampling times of mayonnaise samples

Mayonnaise samples were stored at 20 °C for 4 weeks (28 days) in the dark. The selected storage temperature at 20 °C was choosen according to the shelf life conditions of commercial mayonnaises. During the storage experiment temperature logger was used and regarding the data, temperature range was changing between 17.7 and 19.1 °C.

The mayonnaise samples were distributed in 7 x 300 mL brown colored jars and labelled with the 5 time points for the storage experiment (Week 0, 1, 2, 3 and 4). 120 g of mayonnaise added into each jar in order to make the storage experiment.

At the beginning of the storage experiment and after each week samples were collected for the further analysis Table 3.4 shows the time table of analysis applied.

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Table 3.4: Analysis plan according to weeks.

Date	Storage Time [week]	DSD	PV	Volatiles	FAME	Toco	Sensory
03.04	0 w		X	X	X	X	X
10.04	1 w	X	X	X			
17.04	2 w		X	X			
24.04	3 w		X	X			
01.05	4 w	X	X	X	X	X	X

To determine peroxide value, fatty acid composition and tocopherol content of the oil phase of the mayonnaise samples, samples were kept at -40 °C in the dark during at least 22 h and oil phase of the mayonnaise samples were separated according to Jacobsen et al. (1998) which will be described below (section 4.2.8). For the droplet size distribution analysis, samples were taken and kept in the fridge at 4 °C and analysed at the same day which is described below (section 4.2.7). Around 25 g of the mayonnaise samples were kept at -40 °C in the dark for the secondary volatile compound analysis (section 4.2.12). Samples for the sensory analysis were kept at 4 °C and analysed at the same day which is described below (section 4.2.13).

3.2.9 Droplet size determination

Oil droplets have different sizes and shapes in the emulsion. To be able to describe the average size of the oil droplets, a mathematical model is used to convert it to a sphere and size of the oil droplet can be called as one number which makes it easier to compare. As lipid oxidation occurs on the surface of the oil droplets, Sauter Mean Diameter which is the sphere of equivalent surface area D[3,2] is calculated (Rawle, 1996).

The droplet size was measured in all types of mayonnaise samples by dissolving 1 g mayonnaise in 9 g SDS buffer which was prepared with 10mM NaH_2PO_4, 5 mM SDS, pH 7) (Jacobsen et al., 2001). The mixture was mixed for 30 seconds and placed in utrasonic bath at 30 °C for 20 min. After that mixture was shaken and put in the ultrasonic bath 20 minutes more. Double determinations were performed and

measured 1 time in each mixture using laser diffraction with a Mastersizer2000 (Malvern Instruments, Worcestershire, UK).

The Mastersizer 2000 uses the technique of laser diffraction to measure the size of particles. It does this by measuring the intensity of light scattered as a laser beam passes through a dispersed particulate sample. This data is then analyzed to calculate the size of the particles that created the scattering pattern (Malvern, 2013a). The detection range of the Mastersizer 2000 is between 0.02 and 2000 μm (Malvern, 2013b).

The instrument was set up with a refractive index of material of 1.4694 (sunflower oil) and of dispersant of 1.3333 (water). Mayonnaise – SDS buffer solution was added directly in the recirculating water by dripping until reaching the obscuration between 12-14% at 3000 rpm. The results are given in surface area mean diameter.

3.2.10 Oil phase separation of the mayonnaise

During storage experiment of mayonnaise samples, every week mayonnaise samples were taken and in order to analyse peroxide value, tocopherol content and fatty acid content, oil phase of the mayonnaise was separated by centrifugation. After taking the mayonnaise samples, they were immediately frozen at -40°C for at least 22 hours to separate the emulsion. Before centrifugation, mayonnaise samples were thawed and centrifuged for 10 min at 1620 *g* at 4 °C. After that, oil phase was collected by pipetting and used in the same day or kept in -40°C untill used (Jacobsen et al., 1998).

3.2.11 Fatty acid methyl ester method (FAME)

Fatty acid composition of the mayonnaise samples were determined by applying FAME method based on FF-Analysis Instruction no. 122.01 and detecting them with Gas Chromatography – Flame Ionization Detection (GC-FID) due to the fact that fatty acids require the preparation for subsequent analysis by GC (Cert et al., 2000).

30 mg oil phase of the mayonnaise samples were weighed in methylation glass. For each sample, 100 μL of internal Standard solution (C23:0 Tricosanoic Acid, 2% (w/v) C23:0 i heptan/methanol), 200 μL of heptane with BHT and 100 μL of toluen were mixed and added to the oil samples; then, 1 mL of boron in methanol (BF 3-MeOH) was added in to the methylation glasses. A teflon cap is put on and the lid is screwed on and the samples were mixed for 10 seconds. After that, placed into the

oven (rotor 64) (Anton Paar, Microwave 3000, SOLV). Samples were methylated in the incinerator for 5 minutes at 100 °C and a power of 500 watts. After the methylation of the samples were cooled for 10 minutes which was done automatically in the oven, teflon caps were removed and 1 ml of saturated brine and 0.7 mL of heptane with BHT were added and shaken for 10 seconds. Then mixture was allowed to stand untill separation of heptane occured. Upper part of the separated mixture was pipetted and transferred to a GC vial in order to analyse the fatty acids using GC-FID (Agilent Technologies 7890A, Hørsholm, Denmark) with a column Agilent DB max 127-7012 (0.1 mm ID. Length: 10m. Film: 0.1µm).

3.2.12 Peroxide value

Peroxide value analysis was done according to the photometric method for determination of the peroxide value (PV) by Shanta and Decker (1994) (DTU Food, FF-Analysis Instruction no 121.02). As Fe(II)chloride solution is added on the oil sample Fe^{2+} ions react with the primary oxidation products such as hydroperoxides and form Fe^{3+}. Ammoniumthiocyanate reacts with Fe^{3+} to form a red complex and in this way lipid oxidation and hydroperoxide amount in the sample could be detected by the spectrophotometer.

$$ROOH + Fe^{2+} \longrightarrow RO\bullet + OH^- + Fe^{3+} \quad \textbf{(3.1)}$$

$$Fe^{3+} + 3\ SCN^- \longrightarrow Fe(SCN)_3 \text{ (Red colored complex)} \quad \textbf{(3.2)}$$

20 mg of samples were weighed and overlayed with N_2. Two test tubes were included for the blank samples. 10 mL of chloroform:methanol (7:3) were added into all tubes, overlayed with N_2 and mixed. Another two test tubes were also included for standard samples and 10 mL of Fe(III)-standard-sample which approximately contains 20.7 µg Fe^{3+} were added into the test tubes. To start the reaction, 50 µL of thiocyanate-solution was added into the all test tubes, overlayed with N_2 and mixed. After that, 50 µL of Fe(II)chloride-solution was added into all tubes except for the Fe(III)-standard-sample. Overlayed with N_2 and mixed. Incubated in the dark for 5 minutes. After incubation, all samples were measured at 500 nm, using the spectrophotometer (UV mini1240, Shimadzu, Duisburg, Germany) which was adjusted to 500nm and reset to detect chloroform/methanol (7:3) solvent as zero.

Peroxide value of the samples was calculated according to the formula shown below:

$$PV = \frac{(As - Ab) * k}{55.85 * m * 2} \ meq0_2/kg\ oil \quad \textbf{(3.3)}$$

As= absorbance of sample at 500 nm

Ab= absorbance of reagent-blank-sample at 500 nm

k= k-value (slope) of the standard curve (normally 40.6)

55.85= mole weight of Fe

2= conversion factor from meq oxygen to meq peroxide

m= weight of oil sample in grams

Results were expressed in milli-equivalents peroxide per kg oil (meq O_2/kg).

3.2.13 Tocopherol content

Tocopherol analysis was done according to AOCS Official method (1998) with the name of "Determination of Tocopherols and Tocotrienols in Vegetable Oils and Fats by HPLC", based on FF-Analysis Instruction no. 113.05.

Alpha, beta, gamma and delta tocopherols were quantified by High Performance Liquid Chromatography – Flourescence Detection (HPLC - FLD).

40 mg of oil phase of mayonnaise samples were weighed and mixed with 10 ml of heptane. 1 mL of them were transferred into the vials for HPLC (Agilent 1100 Series, Agilent Technology, CA, USA) analysis. Oil with heptane mixtures and analysis on HPLC were in duplicates.

Standard solution was run in order to know the retention times of the tocopherols and peak areas were compaired to quantify the tocopherol content of samples. HPLC separated the tocopherols based on their size when the prepared heptane-sample mixture was pumped at high pressure through the column containing a separating medium. After tocopherols are separated they went through the FLD where they were detected through state transformation. FLD sends beam of light towards the compounds untill they reach the excited state. After that compound went to the ground state from the excited state by sending out light which is named fluorescence emission. The light sent out was used to detect the presence and amount of the tocopherol content (Frankel, 1998).

3.2.14 Dynamic headspace collection

Dynamic headspace collection of volatile compounds were analysed based on FF-Analysis Instruction no. 731.03.

Before using Tenax tubes containing Tenax GR™, they were regenerated by heating them up to 340°C for 3 minutes. To prepare the Dynamic Headspace System (DHS),

4 g of sample was weighed in purge bottle together with 0,03 g internal standard and values were written down. As shown in the Figure 3.3, purge head was mounted to the nitrogen source, the other edge of the purge head was connected to the S-tube which includes 0.1 g of KOH. S-tube was connected to Tenax GR™ tube. When this system was ready, purge bottle which includes the sample and internal standard was mounted to the purge head and nitrogen flow (150 mL/min) was checked from then open edge of the Tenax GR™ tube. Purge bottle was placed in the water bath at 45°C for 30 minutes. After 30 min, Tenax GR™ tube was disconnected and blown through with nitrogen for 20 min in order to remove water. After that, tube caps were trapped to the Tenax GR™ tube and tubes were placed in autosample collector with automatic thermal desorber (ATD-400, Perkin-elmer, Norwalk, CT) connected to the GC-MS (HP 5890 IIA, Hewlett-Packard, Palo Alto, USA; HP 5972 mass selective detector, Hewlett-Packard, Palo Alto, USA) with column J&W Scientific DB-1701 (30m, I.D. 0.25mm, film 0.5 μm).

First, an empty Tenax GR™ tube was run and then a standard control tube. Second, all the samples were run and standard control tubes were run after every 20 tubes. After all samples were done, volatile compunds were identified and according to their peak heights, calibration curves were performed with external standards in order to quantify the selected volatile compounds.

The volatile compounds were desorbed at 200°C from Tenax GR™ tube by the autosampler collector and then catched by a cold trap. After that, volatile compounds were transferred to the GC and separated. The name of the GC program which is used for separating compounds was called “MAYO02”. Volatile compounds were identified by MS.

The program MAYO02 starts at 35°C for 3 min. Next, the temperature was increased with the flow rate of 3°C/min until 120°C was reached. After that, temperature was increased with the flow rate of 7°C/min until 160°C was reached. At last, the temperature was increased up to 200°C with the flow rate of 15°C/min and held for 4 minutes. The autosampler collectors setting details; inlet split: 9.2 psi, outlet split: 5.0 ml/min desorption flow: 60 ml/min. The GC-MS gas flow was helium with a rate of 1.3 ml/min. The volatile was identified using Chem. station with the library Wiley 138K (John Wiley and sons, Hewlett-Packard, Palo Alto, USA).

To make the calibration curves, 1-penten-3-one, pentanal, 1-penten-3-ol, 1-butanol, 3-methyl, 1-pentanol, hexanal, 2,4-heptadienal, nonanal compounds were selected.

Stock solution was prepared by weighing 0,03 mg of these volatile compounds and diluting the stock solutions to 0.05, 0.025, 0.01, 0.005, 0.0025, 0.001 mg/g. These standard solutions were injected with the amount of 1 µL directly into the Tenax tubes and then analysed by the same method.

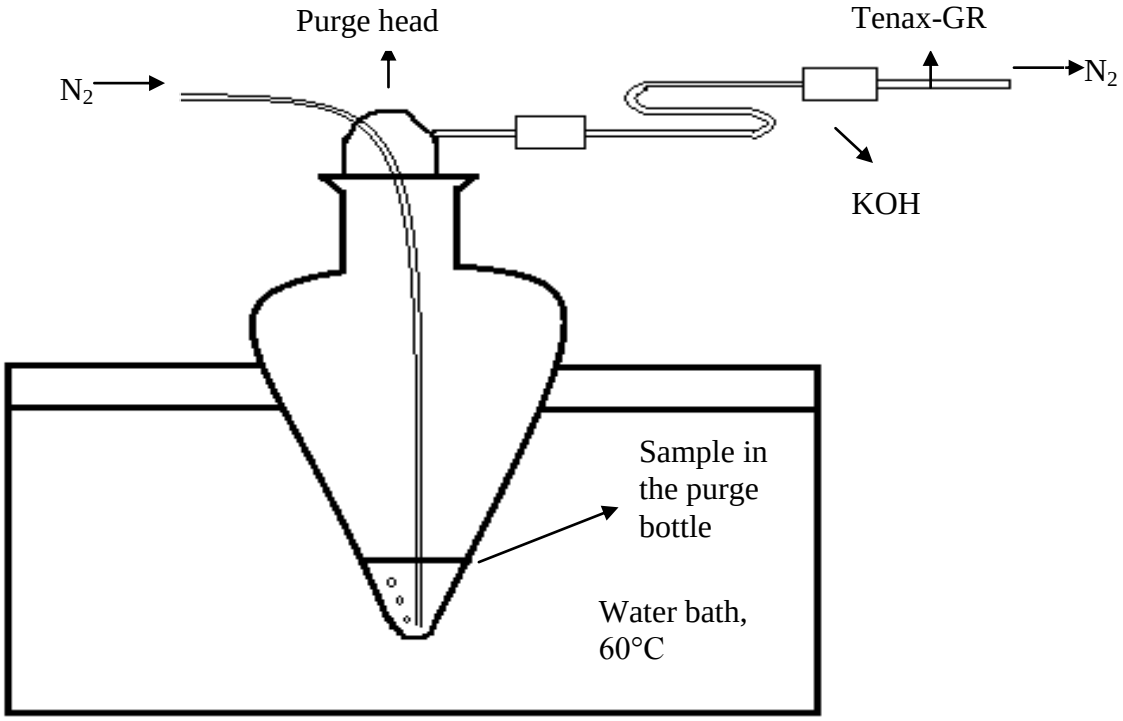

Figure 3.3: Dynamic headspace collection of volatile compounds.

3.2.15 Simple sensory evaluation

Sensory analysis held by a small group which consist of 3 panelists to determine if the oxidation can be detected by a sensory evaluation. Samples were taken and analysed at the same day or kept at 4°C until analysed. Mayonnaise samples were smelled considering fishy/rancidity, acidity, others such as metallic smell. Also consistency was determined with the help of a spoon. Analysis was performed at the beginning (Week 0) and at the end (Week 4) of the storage experiment.

3.2.16 Statistics

The data was analyzed on a 0.05 significant level using a two-ways-ANOVA with a post Bonferroni test. The program Prism 4.0 (Graphpad, La Jolla, USA) was used.

4. RESULTS AND DISCUSSION

In this chapter, all the data found will be shown and discussed. Results and discussion chapter was divided into four part which are:

- Preliminary characterization of F.vesiculosus extracts and fractions,
- Total phlorotannin content of *F. vesiculosus* extracts before/after *in vitro* gastrointestinal digestion system,
- Antioxidant activity of *F. vesiculosus* extracts before/after *in vitro* gastrointestinal digestion system,
- Effect of *F. vesiculosus* extracts on oxidation stability in fish oil enriched mayonnaise

4.1 Characterization of *F. vesiculosus* purified extracts and fractions

Characterization of *F. vesiculosus* purified extracts and their fractions were found as demonstrated in the Table 4.1. After the separation of purified extracts by using three different SPE cartridges (Amino, Diol and Oasis Max), samples run using LC-MS. We extracted ion chromatograms between the range of m/z 300 up to m/z 1000 due to the fact that phlorotannins have the mass covered in this range (Ferreres et al, 2012). Four different phlorotannins were identified according to their mass values in Diol fractions, however, Amino and Oasis Max did not have any of the known phlorotannin mass values.

Table 4.1: Comparison of areas for different fractions.

Comparison of Areas for different fractions								
$[M+H]^+$	375,0712	623,1028	499,09	375,0702	623,1029	623,104	623,1031	747,1194
Retention Time	1,89	2,12	2,43	2,75	3,04	3,12	3,29	3,78
CE1-EA	5276507	3782042	20888760	3772745	4260351	12101927	3346983	
DF1			9429983			2958413		
DF2			11407952		1331301	4907030	1003582	
DF3			4483193		1015111	3850543	752600	
DF4			1046463			1587682		518012

$[M+H]^+$ data of compounds which was found and their areas were shown in the Table 4.1. This table shows that which compound takes place in which diol fractions. The accuracy of the found mass values were checked and showed in the Table 4.2.

Table 4.2: Accuracy of the found mass results comparing with the accurate mass results.

$[M+H]^+$	**Accurate Mass Results**	**Found Mass**	**Accuracy (ppm) (~1,5<)**
375	375,0711	375,0712	0,27
623	623,1031	623,1028	0,48
499	499,0871	499,09	5,81
375	375,0711	375,0702	2,4
623	623,1031	623,1029	0,32
623	623,1031	623,104	1,44
623	623,1031	623,1031	0
747	747,1192	747,1194	0,27

Phlorotannin compounds found in the diol fractions were shown in the Figure 4.1, namely Fucophloroethol A (Molecular Weight: 375.0711 g/mol), Fucodiphloroethol G (MW: 499.0871 g/mol), Fucotriphlorethol A (MW: 623.1031 g/mol), Trifucodiphlorethol A (MW: 747.1192 g/mol).

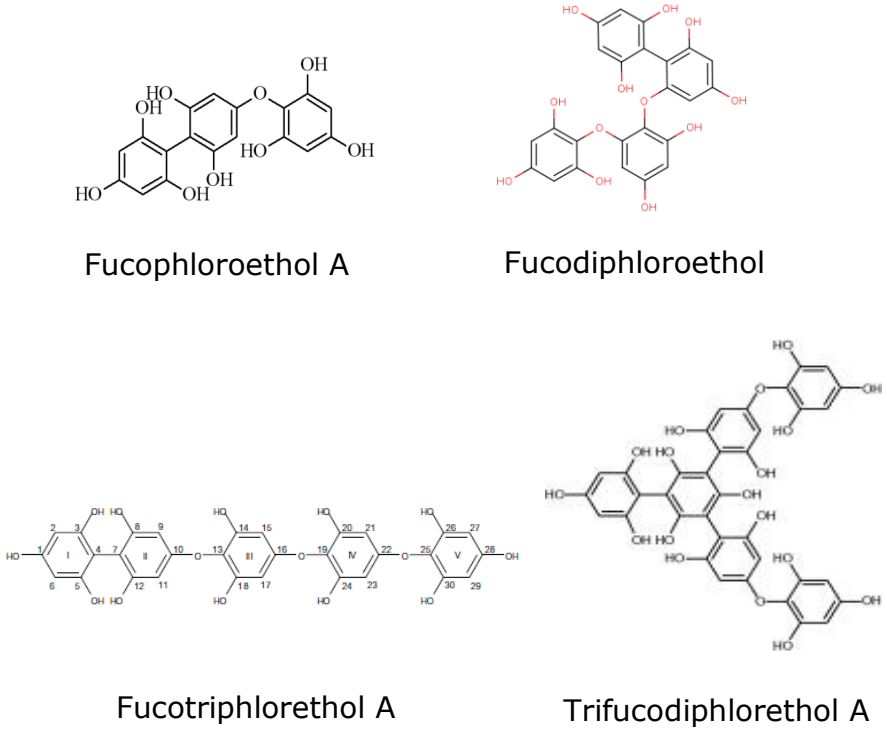

Figure 4.1: Phlorotannin compounds found in the diol fractions of the purified extracts of *F. vesiculosus*.

These phlorotannins were also identified by other studies, for instance; fucophloroethol A (Ferreres et al., 2012; Liu and Gu. 2012), fucodiphloroethol G (Ferreres et al., 2012), fucotriphlorethol A (Parys et al., 2010), trifucodiphlorethol A (Parys et al, 2010; Liu and Gu, 2012).

4.1.1 Antioxidant activity of *F. vesiculosus* fractions

4.1.1.1 DPPH radical scavenging activity of *F. vesiculosus* fractions

As seen in the Figure 4.2, purified ethylacetate extract and diol fractions have high DPPH radical scavenging activity except for the last fraction (DF5) which did not include any phlorotannin when it was identified by LC-MS.

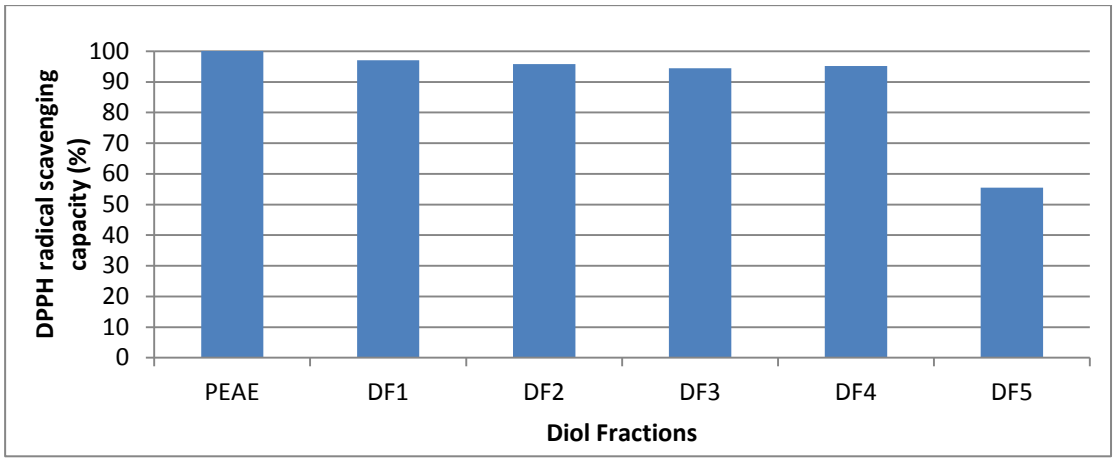

Figure 4.2: DPPH radical scavenging activity of *F. vesiculosus* fractions.

Results were in correlation with total phlorotannin content of *F. vesiculosus* extracts.

4.1.1.2 Reducing power of *F. vesiculosus* fractions

Purified ethylacetate extract and DF1 and DF4 showed higher reducing activity comparing to others. DF5 showed very low reducing power due to the fact that it did not include any identified phlorotannin by LC-MS analysis (Figure 4.3).

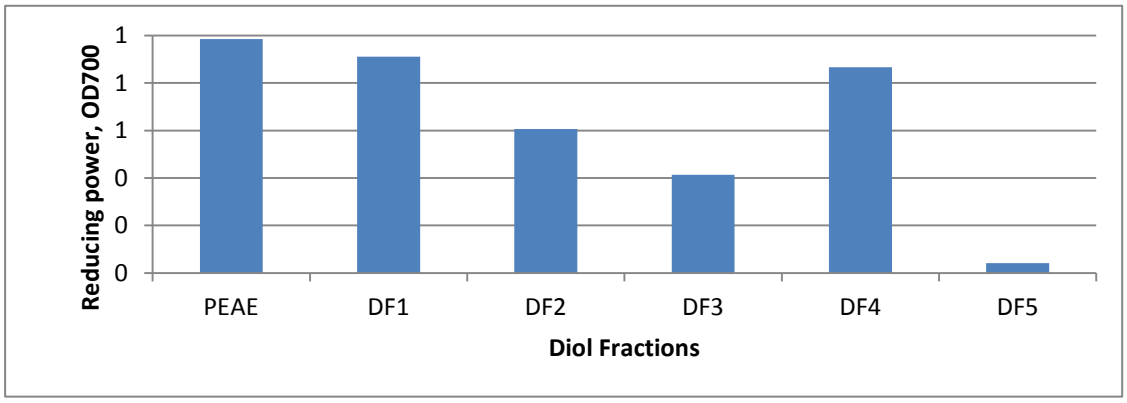

Figure 4.3: Reducing power of *F. vesiculosus* fractions.

4.1.1.3 Metal chelating activity of *F. vesiculosus* fractions

Metal chelating activity of purified ethylacetate extract was interestingly found lower than DF1, DF2 and DF4 and DF5 (Figure 4.4).

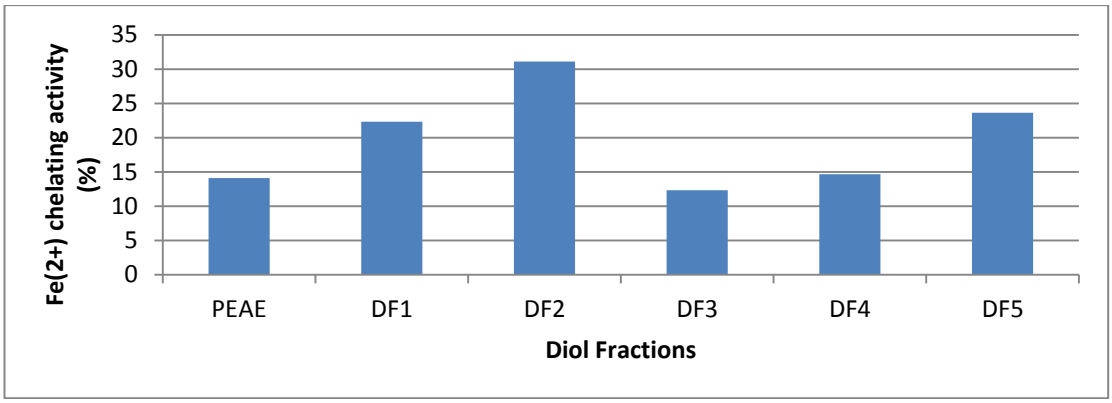

Figure 4.4: Metal chelating activity of *F. vesiculosus* fractions.

4.2 Total Phlorotannin Content (TPC)

4.2.1 TPC in undigested extracts

Total phlorotannin content of water and ethylacetate crude extracts of *F. vesiculosus* were determined as a result of grams of phloroglucinol equivalents (PGE)/100 g of extract. After calibration curve was finished, different concentrations of water and ethylacetate extracts of *F. vesiculosus* samples were calculated according to the calibration curve of phloroglucinol. Absorbances between the calibration curve range were calculated and average of them was determined as the final result as shown in Table 4.3.

Table 4.3: TPCs of *F. vesiculosus* extracts.

Extract Type	TPC (g PGE/ 100 g extract)
WE (Water Extract)	28.5 ±7.1
EAE (Ethylacetate Extract)	45.3 ±4.6

Water extracts and ethylacetate extracts of *F. vesiculosus* were found to have 28.5 and 45.3 g PGE/ 100 g of extract, respectively. Wang et al. (2009) reported that TPCs of water extracts and ethyacetate extracts of *F. vesiculosus* were found 26.3 and 36.5 PGE/ 100 g, respectively. Hence, our results are in accordence with previous data.

4.2.2 TPC in digested extracts

After applying *in vitro* gastrointestinal method, IN, OUT and PG fractions were taken and analysed using total phlorotannin content method in order to determine the amount of the phlorotannins passed through the gastrointestinal digestion system (Table 4.4).

Table 4.4: Bioavailable total phlorotannin content of water and ethylacetate extracts of *F. vesiculosus*.

Fraction	Extract	Average (g PGE/100g)	Standard deviation
IN	**WE**	1.9	0.2
	EAE	2.7	0.3
OUT	**WE**	22.1	1.2
	EAE	63.7	3.1
PG	**WE**	5.1	0.4
	EAE	13.0	0.3

Phlorotannin content of water extract of *F. vesiculosus* was determined as 28.5 g PGE/ 100 g. IN fraction, representing the material which entered the serum (dialyzable fraction), was determined as 1.9 g PGE/100 g WE which means that 5.9% of the total amount of phlorotannins were passed through the dialysis tube which mimics small intestine. Total phlorotannin content of ethylacetate extract of *F. vesiculosus* was found 45.3 g PGE/ 100 g and IN fraction was determined as 2.7 g PGE/ 100 g EAE which means that 6.8% of phlorotannins were passed through the dialysis tube. Both WE and EAE of *F. vesiculosus* thus showed similar bioavailability performance by passing through the dialysis tube with 5.9% and 6.8%, respectively. However, PG fractions which represents the sample taken from gastric digesta (Post Gastric) were found lower than expected. This might be because of the insufficient dilution of the fractions before applying the total phlorotannin content method even though fractions were diluted 10 times with water. High values of OUT fractions representing the material that remained in the GI tract (undialyzable fraction) could be explained by the additional time of extraction (plus 2 h) and/or the effect of intestinal digestive enzyme (pancreatin) on the complex food matrix, facilitating the release of phenolics bound to the matrix (Bouayed *et al*., 2011). Additionally, high results might be due to the 1:50 (OUT fraction: water) dilution was done before total phlorotannin content method was applied.

4.3 Antioxidant Activity

Antioxidant activity of water and ethylacetate crude extracts was determined by using DPPH radical scavenging capacity, reducing power and metal chelating activity assays.

4.3.1 DPPH radical scavenging activity

4.3.1.1 DPPH radical scavenging activity in undigested extracts

Results of water and ethylacetate extracts were compared with butylated hydroxyl toluene (BHT) as a standard and had slightly higher results than the standard.

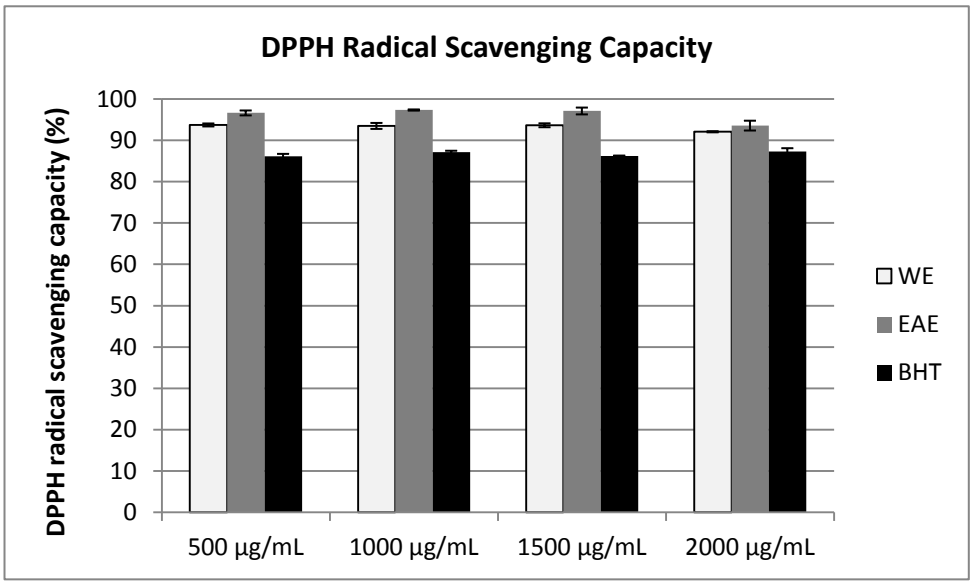

Figure 4.5: DPPH radical scavenging capacity of water extracts and ethylacetate extracts of *F. vesiculosus*.

As shown in the Figure 4.5, ethylacetate extracts of *F. vesiculosus* showed significantly higher DPPH radical scavenging activity for all concentrations comparing to water extracts, which correlates with the TPC of the extracts. Wang et al. (2009) observed that the extracts containing high levels of TPC were also potent DPPH radical scavenger, suggesting that algal polyphenols may be the principal constituents responsible for the antiradical properties of the extracts. Farvin and Jacobsen (2013) reported that the most effective seaweed water extracts were *F. vesiculosus* and *F. serrates* with an 8.3 µg/ml DPPH radical scavenging activity (EC50) in 16 species.

4.3.1.2 DPPH radical scavenging activity in digested extracts

When looking at the PG, IN and OUT fractions, collected during *in vitro* gastrointestinal digestion system was applied for both water and ethylacetate extracts and difference between results of these 2 extracts were not found significantly different from each other (Figure 4.6). DPPH radical scavenging activity found in the PG fractions which was collected after the "gastric phase" was significantly higher than IN and OUT fractions. DPPH radical scavenging activity in IN fractions,

which were collected after passing through dialysis tube mimicking small intestines, was low, however, total phlorotannin contents of IN fractions were also found to be low, namely 5.9% and 6.8% of total amount detected in the water and ethylacetate extracts, respectively. Moreover, it is known that there is a correlation between the DPPH scavenging capacity and total phlorotannin content of the extract (Wang et al., 2009).

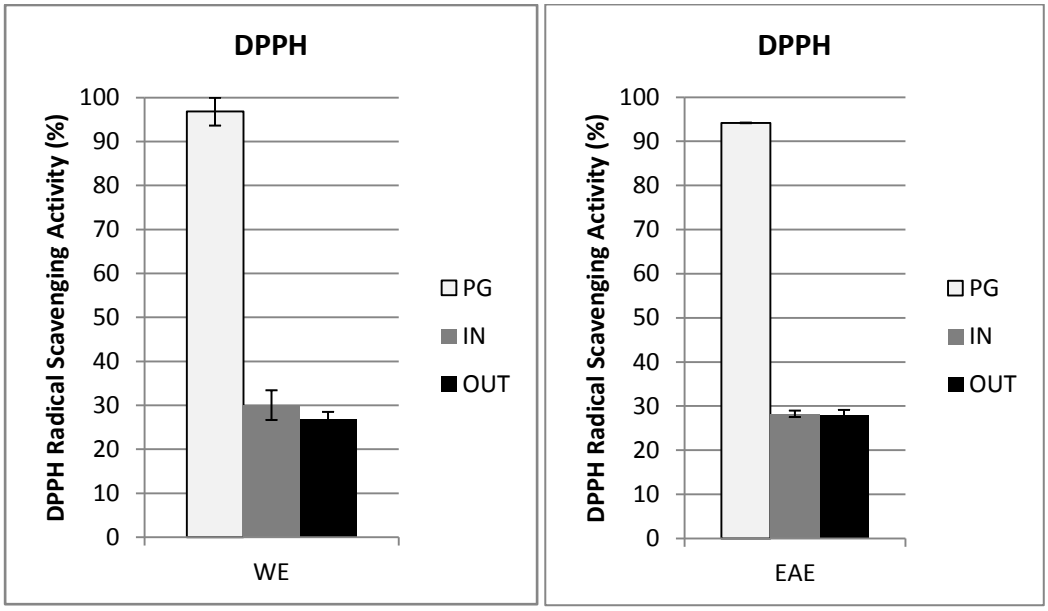

Figure 4.6: DPPH radical scavenging activity of water and ethylacetate extracts after the application of *in vitro* digestion method (PG, IN and OUT fractions).

After the application of *in vitro* digestion method, PG, IN and OUT fractions obtained from water and ethylacetate extracts were statistically similar when we compare water and ethylacetate results. For both extracts, PG fractions, which represents samples taken after gastric digestion, showed high radical scavenging activity but this actvity was lost down to 30% after going through the intestines.

4.3.2 Reducing Power

4.3.2.1 Reducing power in undigested extracts

Reducing results of water and ethylacetate extracts were found high as much as the standard antioxidant ascorbic acid.

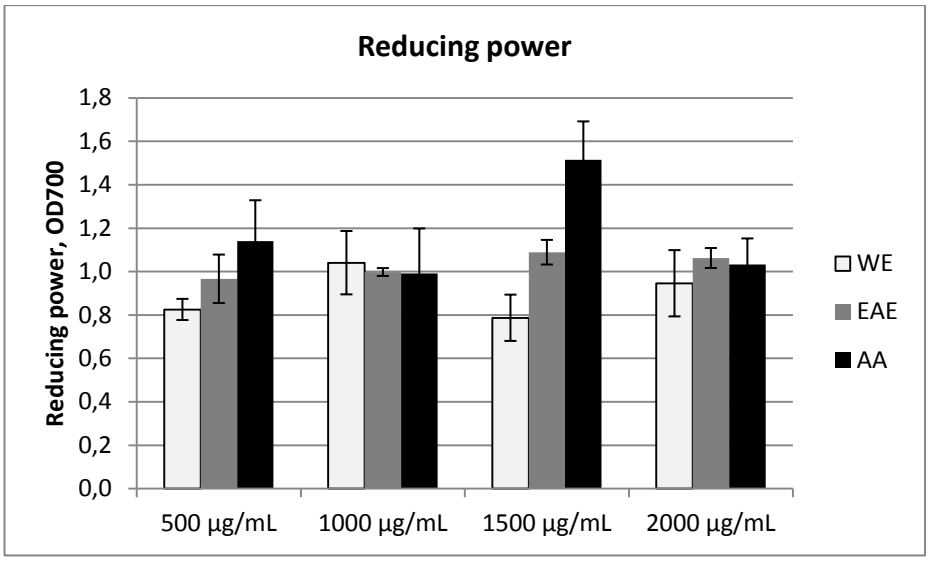

Figure 4.7: Reducing power of water extracts and ethylacetate extracts of *F. vesiculosus.*

Comparing the results of water and ethylacetate extracts, between the concentrations of 500, 1500 and 2000 µg/mL, ethylacetate extracts were slightly higher than water extracts for 500 and 2000 µg/mL, however difference between the extracts at the concentration 1500 was significantly high as shown in the Figure 4.7. Ethylacetate extracts were found slightly higher than water extracts except for the one with the concentration of 1000 µg/mL. Water extracts with the concentration of 1000 µg/mL were found slightly higher than ethylacetate extracts which is in agrrement with the results reported by Farvin and Jacobsen (2013) which reported that both water and ethanolic extracts of Fucus species showed the highest reducing power, however, water extracts showed more reducing power than water extracts which explained by the authors as more hydrophlic compounds present in the water extracts affect their reducing capacities.

Farvin and Jacobsen (2013) showed that reducing power of water extracts of *F. vesiculosus* was found as 1.5 at 1 mg/ml. According to the same article it was also observed that extracts contain high levels of total phenolics also showed activity in reducing ferric ion, suggesting that polyphenols can be the main components which are responsible for antioxidant activity of the extracts.

4.3.2.2 Reducing power in digested extracts

As shown in the Figure 4.8, reducing power of the IN fractions of ethylacetate extracts was found significantly higher than water extracts. PG and OUT fractions were statistically similar for both water and ethylacetate samples. These results

indicate that ethylacetate extracts show their higher reducing power also after passing through the dialysis tube which was representing the intestines.

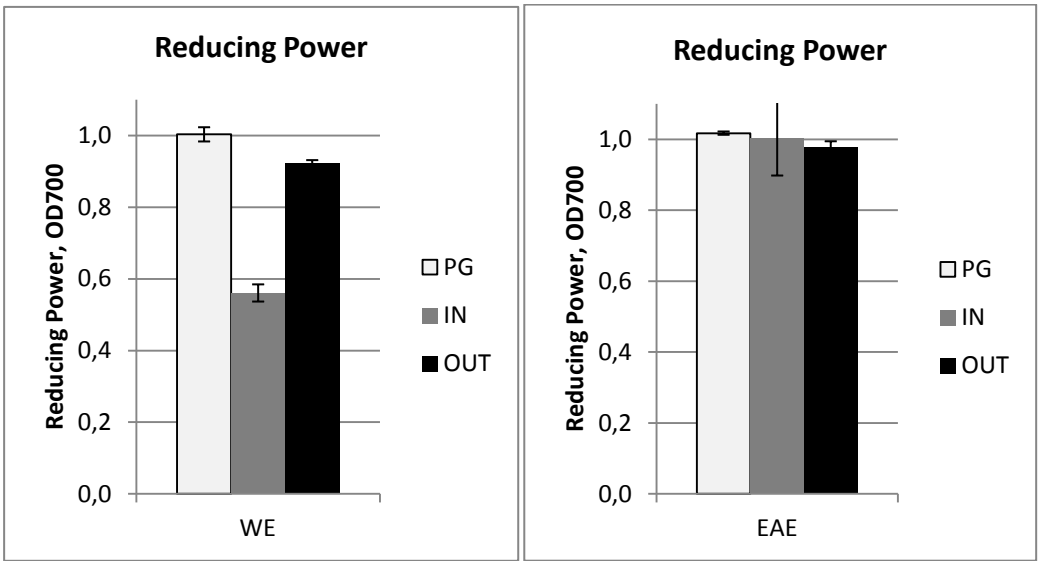

Figure 4.8: Reducing power of water and ethylacetate extracts after the application of *in vitro* digestion method (PG, IN and OUT fractions).

4.3.3 Ferreus ion chelating

4.3.3.1 Ferrous ion chelating activity in undigested extracts

Results of metal chelating activity of water and ethylacetate extracts were compared with EDTA as a standard.

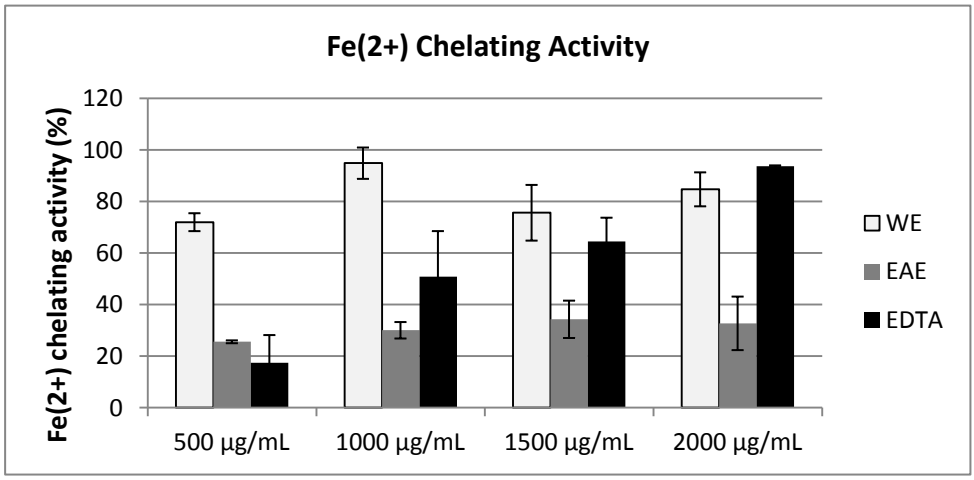

Figure 4.9: Metal chelating activity of water extracts and ethylacetate extracts of *F. vesiculosus*.

Water extracts of *F. vesiculosus* were found significantly higher than ethylacetate extracts at every concentration (Figure 4.9).

Wang et al. (2009) commented that there are contradictory metal chelating results about polyphenols derived from brown seaweeds. Some studies showed that brown

seaweed polyphenols are ferrous ion chelators depending on their unique phenolic structure which includes different numbers of hydroxyl groups in a different localization. On the other hand, some researchers have claimed that metal chelation does not have a role if overall antioxidant activities of polyphenols are considered.

According to Wang et al (2009), water extracts of *F. vesiculosus* showed higher ferreus ion chelating activity than 70% acetone extracts. Wang et al (2012) commented that there was no relationship between total phenolic content and ferreus ion chelating capacity. Farvin and Jacobsen (2013) found that ferreus chelating activity (EC50) of *F. vesiculosus* was 1000.0 and 128.6 μg/ml when extracted with ethanol and water, respectively.

Jacobsen (2010) reported that metal ions have an important role at initiation mechanism for lipid oxidation in fish oil enriched mayonnaise, therefore metal chelators may prevent lipid oxidation. Another study have shown the effects of lactoferrin, phytic acid and EDTA in fish oil enriched mayonnaise, the most effective metal chelator was found as EDTA (Jacobsen et al., 2001b; Nielsen et al, 2004). However, since EDTA is a synthetic antioxidant and there is an increasing trend for using natural antioxidants, better sources are preferred to provide the stability of fish oil enriched mayonnaise. In the present study, water extracts of *F. vesiculosus* samples were found to be high at different concentrations. This may show that water extracts of *F. vesiculosus* can fulfill the need or be an alternative solution for fish oil enriched mayonnaise as will be discussed later.

According to Farvin and Jacobsen (2013), *F. vesiculosus* showed the highest Fe^{2+} chelating activity among 16 Danish seaweeds with the values of 1000.0 and 128.6 μg/ml ferreus chelating activity (EC50) in the ethanol and water extracts of *F. vesiculosus*, respectively.

Moreover, Wang et al. (2009) was reported that water extracts of *F. vesiculosus* showed the highest metal chelating activity (above 95%) among 12 seaweed species.

4.3.3.2 Ferrous ion chelating activity in digested extracts

It was observed that there was no significant differences between the results of PG, IN and OUT fractions of water and ethylacetate extracts as shown in the Figure 4.10.

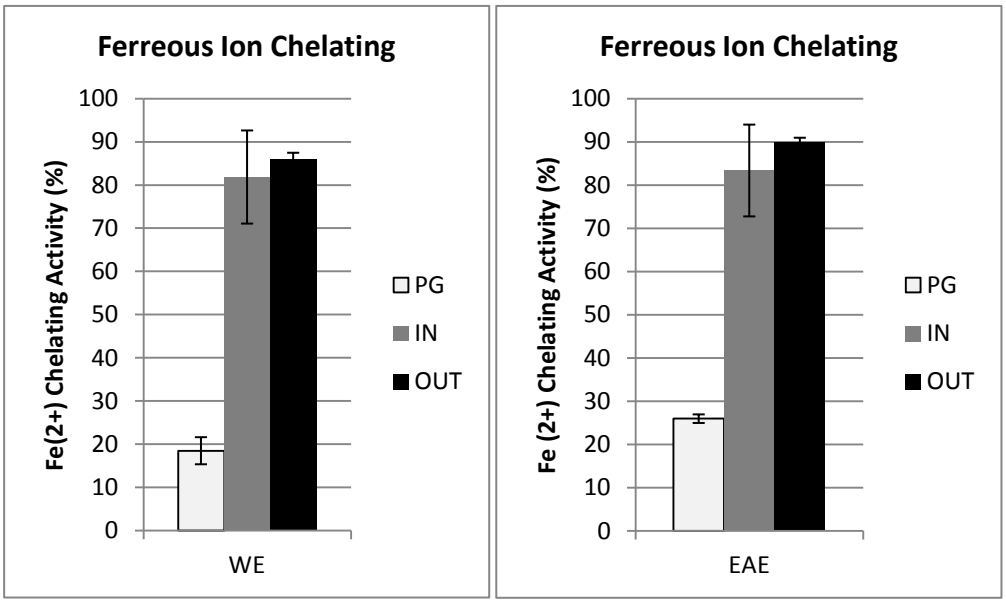

Figure 4.10: Metal chelating activity of water and ethylacetate extracts after the application of *in vitro* digestion method (PG, IN and OUT fractions).

IN fractions showed high metal chelating activity for both water and ethylacetate extracts.

Considering all antioxidant analysis on digested water and ethylacetate extracts showed unexpected results. Normally, PG fraction was expected to be the sum of IN and OUT fractions, however, this was not observed. This might be due to the amount added at the beginning of the *in vitro* gastrointestinal system which was 0.5 g of the extract. According to the method described by McDougall et al. (2005), 5 g of food sample was added into the system to digest but in this study as it was extracts, 0.5 g was decided to be added. However, in order to apply antioxidant assays, fractions needed to be diluted with water up to 50 times and taken 150 and 200 µL of samples for the analysis. Thus, this might create a problem for having the right amount of compounds to see the effect of antioxidant activity.

4.4 Effect of *F. vesiculosus* extracts on oxidation stability in fish oil enriched mayonnaise

4.4.1 Droplet size distribution

Droplet size distribution of the mayonnaise samples were measured as duplicates as shown in the Figure 4.11. There was no significant differences between week 1 and week 4 for all mayonnaise samples. However, significant differences were determined between mayonnaise samples. At week 1, Mayo_EAE2 was found to have significantly larger droplet size than Mayo_ref, Mayo_WE1, Mayo_WE2 and Mayo_EAE1. At week 4, Mayo_WE3 was found to be significantly larger than Mayo_ref, Mayo_WE1 and Mayo_EAE1.

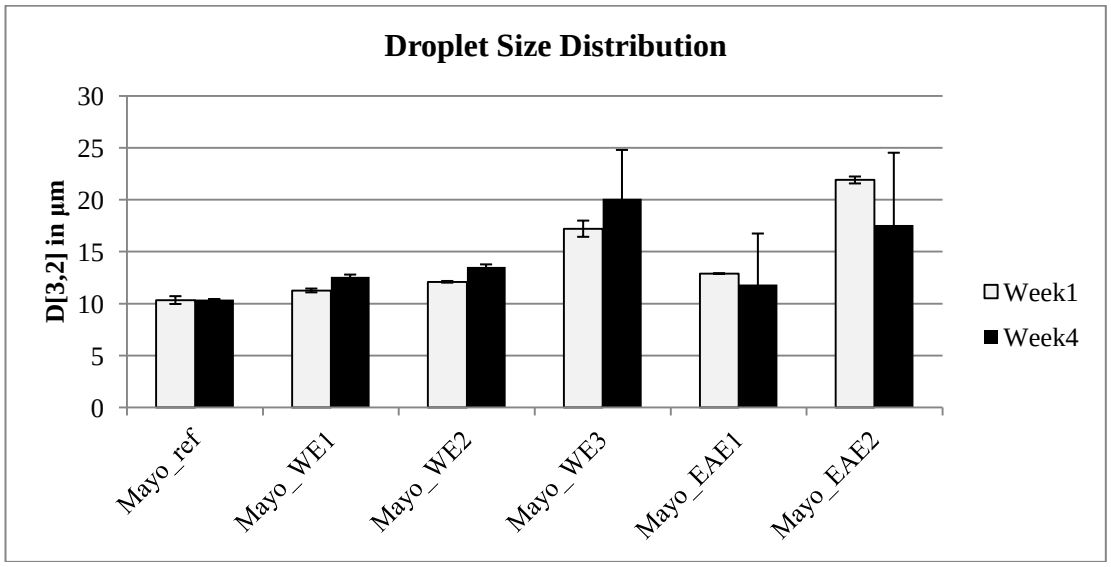

Figure 4.11: The droplet size of the mayonnaises in D[3,2] at week 1 and 4 (n=2).

Results showed that droplet size of the mayonnaise with WE of *F. vesiculosus* was increasing with the increasing concentration of WE, but they were not significant from each other at the end of one week. According to the results, at week 4, Mayo_WE3 was found to be significantly larger than referance (Mayo_ref) and mayonnaise sample with 1 g of water extract of *F. vesiculosus* (Mayo_WE1). This may be because of the increasing amount of extract in the mayonnaise sample which indicates that droplet size may increase with the added water extract or might be due to the accidentally addition of sugar and salt after mixing the oil and water phase while producing the mayonnaise.

Droplet size results of the mayonnaise samples with ethylacetate extract showed that after first week of the storage, difference between Mayo_EAE1 and Mayo_EAE2 was significantly high but after 4 weeks of the storage experiment it was found that

difference between them was not significant. However, according to the results measured after 1 week of storage experiment shown in Figure 5.7 with red colored columns, it was observed that droplet size was increasing with the increase of extracts' concentration. After 4 weeks of storage experiment, it was seen that mayonnaise samples with water extracts were slightly increased and mayonnaise samples with ethylacetate extracts were slightly decreased.

According to Jacobsen et al. (2000), droplet size of fish oil enriched mayonnaise influenced the lipid oxidation. Results were observed that mayonnaise samples with smaller droplet sizes were oxidised faster at the initial part of the storage period than the ones with larger droplet sizes. However, at the later part of the storage experiment, there was no influence of droplet size on oxidative flavour. This was explained by the effect of large interfacial area of the small droplets that increased the contact area between iron and lipid hydroperoxides which were located in the aqueous phase and interface, respectively, and oxidation rate was increased comparing to larger droplets. At the later stages of storage, oxidation started to occur more into the oil droplets. Thus, droplet size becomes less important in affecting the oxidation rate.

4.4.2 Fatty acid composition

The FAME analysis determines the fatty acid distribution of the mayonnaise samples enriched with fish oil. The results are obtained in % of total fatty acid content. The average fatty acid contents of the mayonnaises at week 0 and 4 are illustrated in Table 4.5.

Table 4.5: Fatty acid composition of mayonnaise samples enriched with fish oil at week 0 and 4.

Fatty acid		**WEEK 0**						**WEEK 4**					
(Area %)	**Fish oil**	**Mayo _ref**	**Mayo _WE1**	**Mayo_ WE2**	**Mayo_ WE3**	**Mayo_ EAE1**	**Mayo_ EAE2**	**Mayo _ref**	**Mayo_ WE1**	**Mayo_ WE2**	**Mayo_ WE3**	**Mayo_ EAE1**	**Mayo_ EAE2**
14:00	3,52	0,63	0,64	0,64	0,63	0,64	0,63	0,65	0,6	0,63	0,63	0,63	0,64
14:01	0,26	0,04	0,04	0,04	0,04	0,04	0,04	0,04	0,03	0,04	0,03	0,04	0,03
15:00	0,32	0,06	0,07	0,06	0,06	0,06	0,06	0,06	0,06	0,06	0,07	0,06	0,06
16:00	9,95	5,44	5,45	5,45	5,41	5,44	5,44	5,51	5,44	5,42	5,44	5,47	5,48
16:1 (n-7)	9,21	1,72	1,74	1,77	1,69	1,72	1,7	1,76	1,63	1,66	1,69	1,7	1,71
16:2 (n-4)	0,41	0,12	0,09	0,09	0,07	0,1	0,08	0,1	0,07	0,09	0,09	0,07	0,07
16:3 (n-4)	0,52	0,12	0,12	0,12	0,12	0,12	0,12	0,12	0,12	0,13	0,13	0,12	0,13
17:00	0,35	0,1	0,1	0,1	0,1	0,1	0,1	0,11	0,1	0,11	0,11	0,1	0,1
16:4 (n-1)	0,29	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05	0,05
18:00	2,04	0	0	0	0	0	0	0	0	0	0	0	0
18:1 (n-9)	16,93	52,08	51,84	52,08	51,95	51,95	51,97	52,11	52,73	52,42	52,11	52,21	52,14
18:1 (n-7)	4,84	2,42	2,54	2,24	2,34	2,29	2,27	2,68	2,52	2,48	2,55	2,5	2,48
18:2 (n-6)	1,9	16,92	16,81	16,76	16,75	16,74	16,76	17,26	17,14	16,89	16,8	16,83	16,79
18:2 (n-4)	0,15	0,04	0,03	0,03	0,03	0,03	0,03	0,04	0,03	0,03	0,03	0,02	0,02
18:3 (n-6)	0,13	0,03	0,03	0,02	0,02	0,03	0,02	0,04	0,04	0,04	0,03	0,03	0,03
18:3 (n-4)	0,93	0,02	0,01	0,03	0,02	0,02	0,02	0,02	0,02	0,02	0,02	0,02	0,02
18:3 (n-3)	0	8,55	8,49	8,44	8,45	8,43	8,45	8,75	8,6	8,44	8,41	8,43	8,41
18:4 (n-3)	2,72	0,48	0,49	0,49	0,48	0,48	0,48	0,47	0,45	0,46	0,47	0,47	0,47
20:00	0	0,5	0,5	0,5	0,51	0,5	0,5	0,46	0,5	0,51	0,51	0,51	0,51
20:1 (n-11)+(n-9)	12,43	3,41	3,51	3,55	3,5	3,52	3,48	3,17	3,26	3,53	3,55	3,38	3,51
20:1 (n-7)	0,29	0,12	0,11	0,11	0,19	0,11	0,11	0,09	0,11	0,08	0,05	0,05	0,05
20:2 (n-6)	0,29	0,11	0,11	0,12	0,11	0,12	0,11	0,11	0,11	0,11	0,11	0,11	0,11
20:3 (n-6)	0,02	0,02	0	0,02	0,02	0,02	0,02	0,01	0	0,02	0,02	0,02	0,02
20:4 (n-6)	0,37	0,1	0,11	0,12	0,1	0,11	0,1	0,11	0,1	0,11	0,11	0,12	0,11
20:3 (n-3)	0,12	0,01	0	0,03	0,02	0,03	0,01	0,01	0	0,01	0,01	0,01	0,02
20:4 (n-3)	0,65	0,12	0,12	0,12	0,11	0,12	0,12	0,11	0,1	0,11	0,11	0,11	0,11
20:5 (n-3) EPA	9,24	1,66	1,7	1,69	1,66	1,67	1,65	1,55	1,49	1,58	1,63	1,61	1,64
22:1 (n-11)	0,78	1,01	1,06	1,06	1,05	1,05	1,04	0,84	0,9	0,99	1,02	1,01	1,03
22:1 (n-9)	5,3	0,4	0,42	0,42	0,43	0,41	0,41	0,36	0,39	0,41	0,42	0,41	0,42
21:5 (n-3)	0,39	0,07	0,08	0,07	0,07	0,07	0,07	0,06	0,06	0,07	0,07	0,07	0,07
22:5 (n-3)	0,99	0,19	0,2	0,2	0,2	0,19	0,19	0,16	0,17	0,19	0,2	0,2	0,2
22:6 (n-3) DHA	10,33	2,01	2,08	2,07	2,02	2,05	2,03	1,71	1,73	1,88	1,96	1,95	1,98
24:1 (n-9)	0,05	0,09	0,09	0,09	0,09	0,09	0,09	0,08	0,08	0,09	0,09	0,09	0,09

Oleic acid (18:1 (n-9)) content of samples were detected between 51.84 and 52.08 at week 0; 52.11 and 54.42 at week 4. Palmitic acid (16:0) content of samples were found between 5.41 and 5.45 at week 0; 5.42 and 5.51 at week 4. 9-Eicosenoic acid and 11-Eicosenoic acid (20:1 (n-11)+(n-9)) were detected between 3.41 and 3.55 at week 0; 3.17 and 3.55 at week 4. EPA (20:5 (n-3)) was found between 1.65 and 1.70 at week 0; 1.49 and 1.64 at week 4. DHA (22:6 (n-3)) was found between 2.01 and 2.08 at week 0; 1.71 and 1.98 at week 4. As shown on the Table 4.3, differences between samples and storage time points were not different than each other, however, EPA and DHA results were decreased after 4 weeks storage experiment. EPA content of Mayo_ref, Mayo_WE1, Mayo_WE2, Mayo_WE3, Mayo_EAE1, Mayo_EAE2 samples were decreased 6.7%, 12.1%, 6.5%, 1.7%, 3.5% and 0.8%, respectively. Loss of DHA content of Mayo_ref, Mayo_WE1, Mayo_WE2, Mayo_WE3, Mayo_EAE1, Mayo_EAE2 samples were 14.9%, 16.5%, 9.1%, 2.9%, 5.0% and 2.2%, respectively. These results showed that omega-3 long-chain polyunsaturated fatty acids are prone to oxidation due to their double bonds. As known, when fish oil oxidize, unstable free radicals and hydroperoxides occur which are prone to further decomposition into products such as aldehydes and ketones (Arab-Tehrany et al., 2012). Also, oxidation of PUFAs produces a mixture of volatile secondary oxidation products which cause off-flavors (Let et al. 2004). Additionally, these data showed water and ethylacetate extracts of *F. vesiculosus* have an effect on preventing EPA and DHA loss during storage. Mayo_WE3 which had the highest content of water extract lost only 0.8% and 2.2% of EPA and DHA content. Mayo_EAE2, Mayo_EAE1 and Mayo_WE2 were the following samples which showed a protection of losing omega-3 fatty acids. Mayo_WE1 lost the highest amounts of its EPA and DHA content which is in agrrement with the results of simple sensory analysis, Mayo_WE1 was reported as it had the highest fishy/rancid odor after 4 weeks of storage experiment in all samples.

4.4.3 Peroxide value (PV)

PV of fish oil used in mayonnaise was determined as 0.16 meq O_2/kg oil. PVs for 4 weeks storage period are shown at the Figure 4.12. The mayonnaise samples had no significant difference in PVs at week 0 (between 0.27 and 0.48 meq O_2/ kg oil).

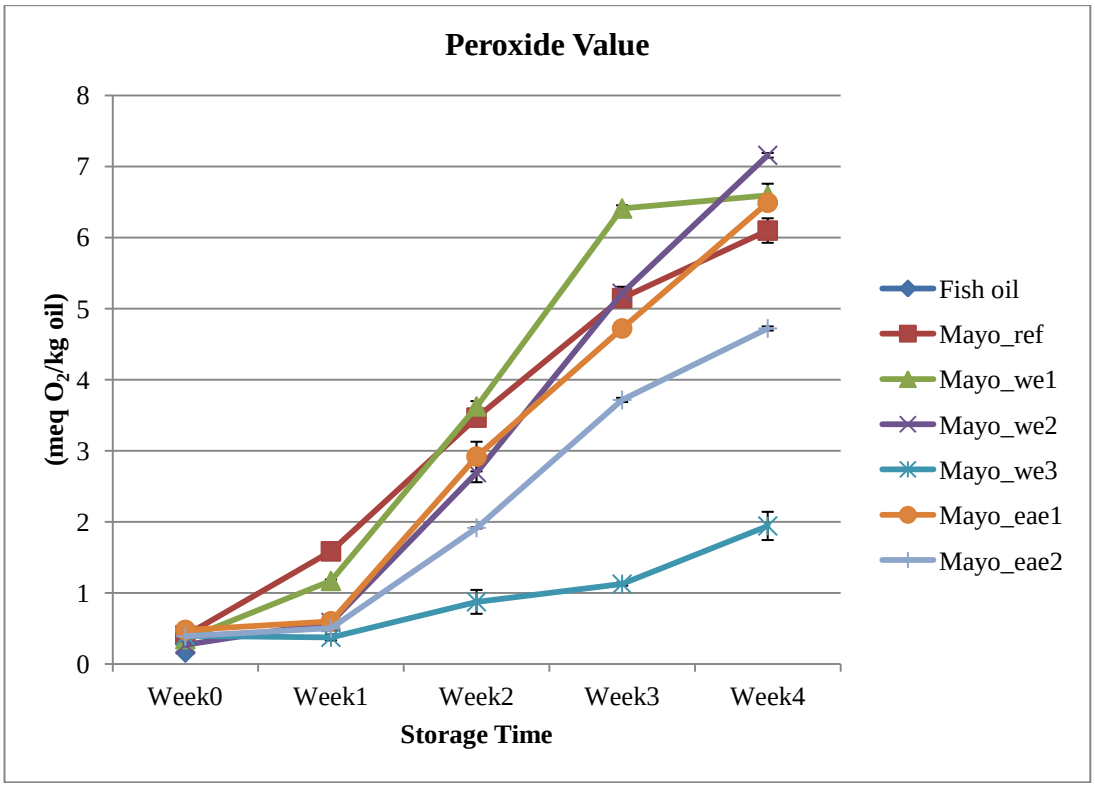

Figure 4.12: The PV during 4 weeks of storage (n=2).

At week 1, Mayo_ref (1.58 meq O_2/ kg oil) and Mayo_WE1 (1.17 meq O_2/ kg oil) were found to be significantly higher than the other samples. At week 2, PV values of Mayo_WE2 (2.69 meq O_2/ kg oil) and Mayo_EAE1 (2.92 meq O_2/ kg oil) were found to be significantly higher than Mayo_WE3 (0.87 meq O_2/ kg oil) and Mayo_EAE2 (1.91 meq O_2/ kg oil). Mayo_ref (3.49 meq O_2/ kg oil) and Mayo_WE1 (3.62 meq O_2/ kg oil) were found to be significantly higher than other mayonnaise samples. At week 3, Mayo_WE3 (1.12 meq O_2/ kg oil) had significantly lower PV than the others. Following lowest PV values were determined for Mayo_EAE2 (3.71 meq O_2/ kg oil) and Mayo_EAE1 (4.72 meq O_2/ kg oil), respectively. At the end of the storage experiment, all the mayonnaise samples were found to be significantly different from each other except from Mayo_WE1 and Mayo_EAE1 in the order of Mayo_WE3 (1.94 meq O_2/ kg oil) < Mayo_EAE2 (4.72 meq O_2/ kg oil) < Mayo_ref (6.1 meq O_2/ kg oil) < Mayo_EAE1 (6.49 meq O_2/ kg oil) < Mayo_WE2 (7.16 meq O_2/ kg oil).

After one week storage, Mayo_ref, Mayo_WE1 and Mayo_WE2 were found to be significantly higher than the beginning of the storage experiment. After 2 weeks, all the samples were significantly higher than the week1 and week0. At the end of 3rd week, all the samples were significantly higher than the previous week except for

Mayo_WE3. At the end of the storage experiment, all the samples were significantly higher than the end of 3rd week except for Mayo_WE1. After all, Mayo_WE3 showed the slowest increase in the PV value which shows that higher concentration of water extracts in comparison with the others showed better effect on inhibiting the primary oxidation products.

When results were evaluated considering the information explained in the section 5.3 (para 4), after one week of the storage experiment, droplet size results of mayonnaise samples with water extracts were ranking in the order of Mayo_ref < Mayo_WE1 < Mayo_WE2 < Mayo_WE3 and PV results were ranked in the order of Mayo_ref > Mayo_WE1 > Mayo_WE2 > Mayo_WE3; samples with ethylacetate extracts were ranking in the order of Mayo_ref < Mayo_EAE1 < Mayo_EAE2 and PV results were ranking in the order of Mayo_ref > Mayo_EAE1 > Mayo_EAE2. These results are in aggreement with the argument stated by Jacobsen et al. (2000b) about the effect of droplet size on oxidation at the initial stages of storage. At the later stages of the storage experiment, droplet size results of the mayonnaise samples with water extracts were observed in the order of Mayo_ref < Mayo_WE1 < Mayo_WE2 < Mayo_WE3 and PV results were ranked in the order of Mayo_WE2 > Mayo_WE1 > Mayo_ref > Mayo_WE3; samples with ethylacetate extracts were ranking in the order of Mayo_ref < Mayo_EAE1 < Mayo_EAE2 and PV results were ranking in the order of Mayo_EAE1 > Mayo_ref > Mayo_EAE2. These results showed that after 4 weeks of storage experiment, droplet size of the samples did not affect the oxidation rate as much as oxidation was affected by droplet size at the initial stages, in aggreement with the study done by Jacobsen et al (2000b).

According to Jacobsen (1999), mayonnaise with larger droplet sizes had lower PVs, results in present study showed that Mayo_WE3 and Mayo_EAE2 had higher droplet size values and also had lower PVs comparing with the other mayonnaise samples after 4 weeks of the storage experiment.

4.4.4 Tocopherol content

Total tocopherol content of all samples were found to be increased except for referans sample (Mayo_ref), however these increase/decrease were not statistically significant between week 0 and week 4 as shown on the Figure 4.13. Likewise, no significant difference between mayonnaise samples was determined at week 0 and 4.

Total tocopherol results were found between 500.40 and 533.08 µg toc/ g oil during the storage experiment. It was reported that if the tocopherol content is higher than 590 mg/kg oil in fish oil enriched mayonnaise, it may have a prooxidative effect (Jacobsen et al., 2000a; Jacobsen and Nielsen, 2007). Results found in our study were below this value which means that they did not have a prooxidative effect.

Total tocopherol content of fish oil used in the mayonnaise was determined as 392.78 µg toc/ g oil. Its alpha tocopherol, beta tocopherol, gamma tocopherol and delta tocopherol contents were found as 234.46, 3.65, 109.27, 45.40 µg toc/ g oil, respectively. According to Jacobsen et al. (2008), total tocopherol concentration in fish oil was found 304 µg toc/ g oil.

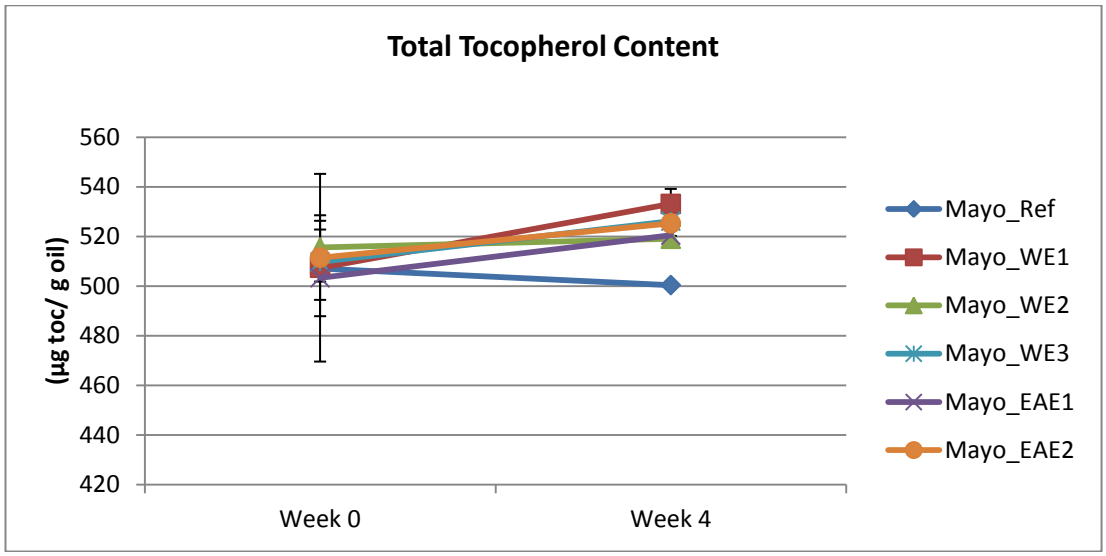

Figure 4.13: Total tocopherol content of mayonnaise samples at week 0 and 4.

Alpha tocopherol content of mayonnaise samples were determined as not statistically different between week 0 and week 4 (Figure 4.14). No significant difference was identified between all mayonnaise samples at the beginning of the storage experiment (week0), however at the end of the storage experiment alpha tocopherol content of Mayo_ref were determined as significantly lower than Mayo_WE3 and Mayo_EAE2. Alpha tocopherol results were found in the range of 190.63-195.24 and 177.46-195.56 for week 0 and week 4, respectively.

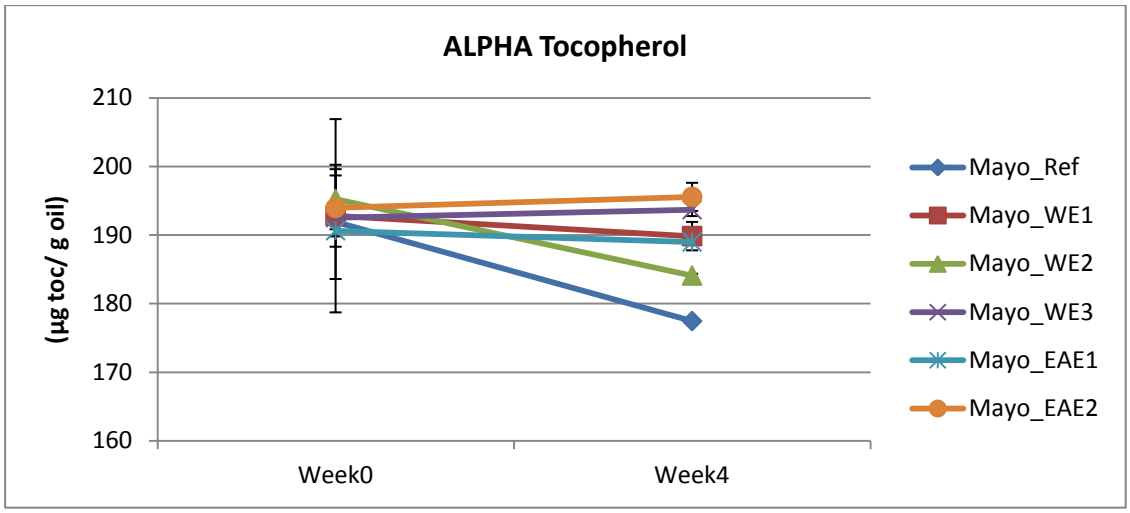

Figure 4.14: Alpha tocopherol content of mayonnaise samples at week 0 and 4.

When considering the effects of added concentrations of *F. vesiculosus* extracts, amounts of decrease were seen in the order of Mayo_ref > Mayo_WE2 > Mayo_WE1 > Mayo_EAE1 and increase were seen for two samples, in the order of Mayo_EAE2 > Mayo_WE3. These results may explain that higher concentrations of *F. vesiculosus* extracts have a protective effect on alpha tocopherol content. However, sample with water extract of *F. vesiculosus* (Mayo_WE2) did not show the same effect as the sample with ethylacetate extract (Mayo_EAE2) did, even though they had the same concentration of extracts (1.5 g/ kg). Moreover, Mayo_ref was degraded faster than the other samples, it might show that *F. vesiculosus* extracts have protective effects on alpha tocopherol degradation.

Similar results were reported by Larsen and Jacobsen (unpublished data), alpha tocopherol content of reference sample of fish-oil enriched milk was decreasing more than the other samples including *F. vesiculosus* extracts. Moreover, fish oil enriched milk samples with ethylacetate extracts were decreased in small amounts comparing to the water extracts.

Alpha tocopherol has the highest bioactivity due to the fact that it is a chain-breaking lipid soluble antioxidant. Temperature, lipid composition, phsical state and concentration designate its behaviour as an antioxidant or a prooxidant (Arab-Tehrany et al., 2012).

As shown on the Figure 4.15, beta tocopherol content of the samples was found in the range of 49.99-52.29 and 58.38-62.33 for week 0 and week 4, respectively. The increase in the amount of the beta tocopherol content between week 0 and week 4 was found to be statistically significant. At week 0, differences between beta

tocopherol values of all samples were found statistically not significant. Similarly, no significant difference was found between samples after storage experiment was done. This significant increase between week 0 and 4 can be explained by the reason that the equipment might show a measuring error during the analysis.

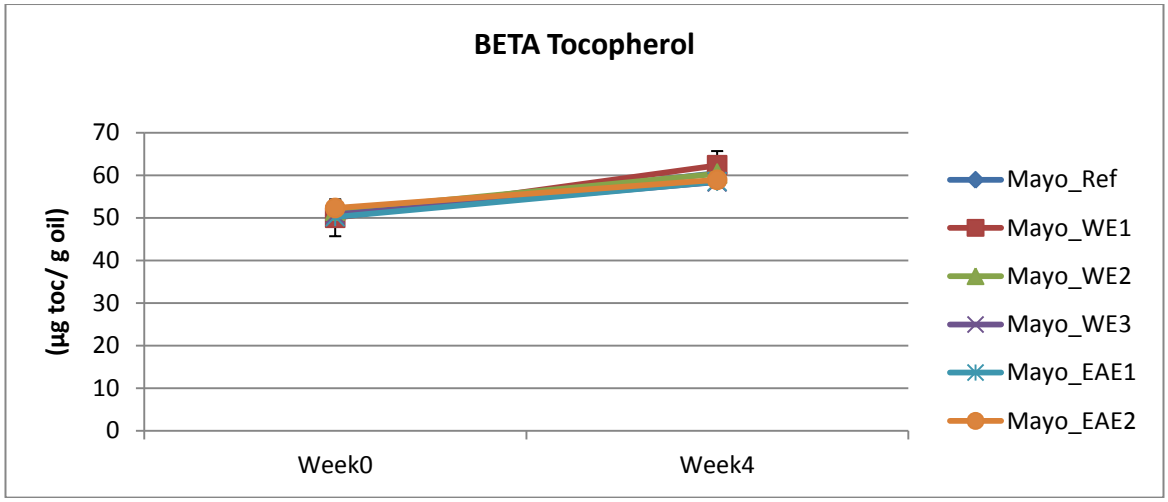

Figure 4.15: Beta tocopherol content of mayonnaise samples at week 0 and 4.

As shown on the Figure 4.16, gamma tocopherol content of the samples was found in the range of 247.08-252.88 and 244.90-266.27 for week 0 and week 4, respectively. According to Jacobsen et al. (2005), good antioxidative effect of reape seed oil is linked between its high gamma tocopherol amount. The increase in the amount of the gamma tocopherol content between week 0 and week 4 was not found statistically significant. At week 0, differences between gamma tocopherol values of all samples were not found statistically significant. However, after 4 weeks Mayo_ref was found significantly lower than Mayo_WE1.

Rape seed oil was known with its high tocopherol content, especially gamma tocopherol. It was discussed by Jacobsen et al. (2010), if additional gamma tocopherol will have an effect in fish oil enriched mayonnaise (16% of fish oil and 64% of rape seed oil) which is an emulsion system. However, it was reported that tocopherol already present in the oils used in the mayonnaise, therefore, it has a poor effect as an antioxidant. Moreover, due to the fact that deterioration reactions between omega-3 PUFA and the formed radicals from the decomposed peroxides will occur at the o/w interface, tocopherol can be active at a limited level (Jacobsen et al., 2010).

Results showed that antioxidant addition showed a protective effect on gamma tocopherol content. Mayo_ref was the only sample which was decreased after 4

weeks storage period. This might be due to the fact that *F. vesiculosus* extracts have a protective effect on gamma tocopherol degradation.

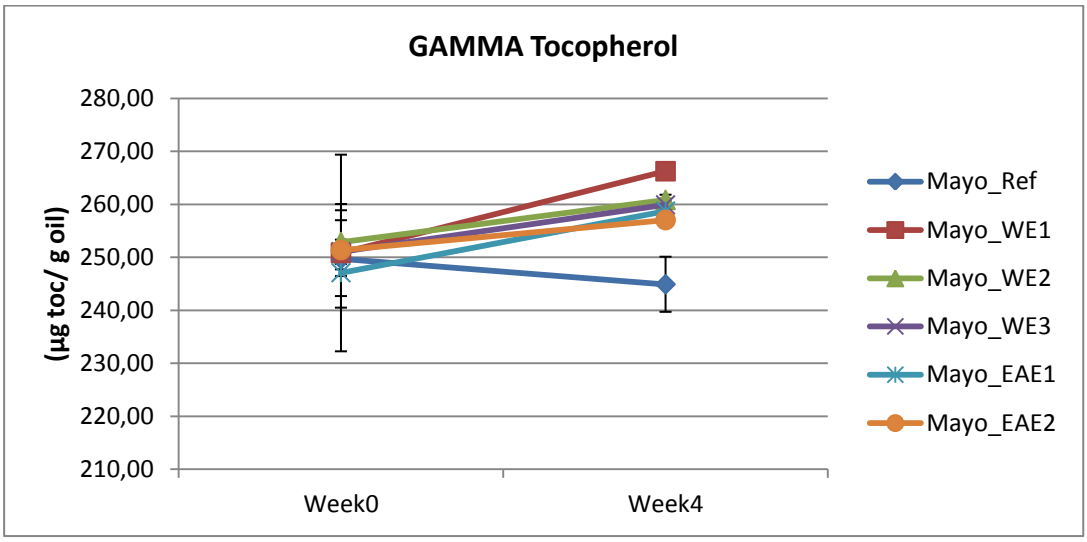

Figure 4.16: Gamma tocopherol content of mayonnaise samples at week 0 and 4.

Delta tocopherol content of the samples was found in the range of 13.77-15.87 and 13.76-17.52 at week 0 and week 4, respectively (Figure 4.17). Differences between week 0 and week 4 was not found statistically significant. At week 0, differences between samples were not significantly different, however at the end of the storage experiment, Mayo_ref was found significantly higher than the other samples. Mayo_ref and Mayo_WE1 were increased with the amount of 2.60 and 0.90, respectively while the other samples were slightly decreased. This might be due to the measuring errors originated from equipment.

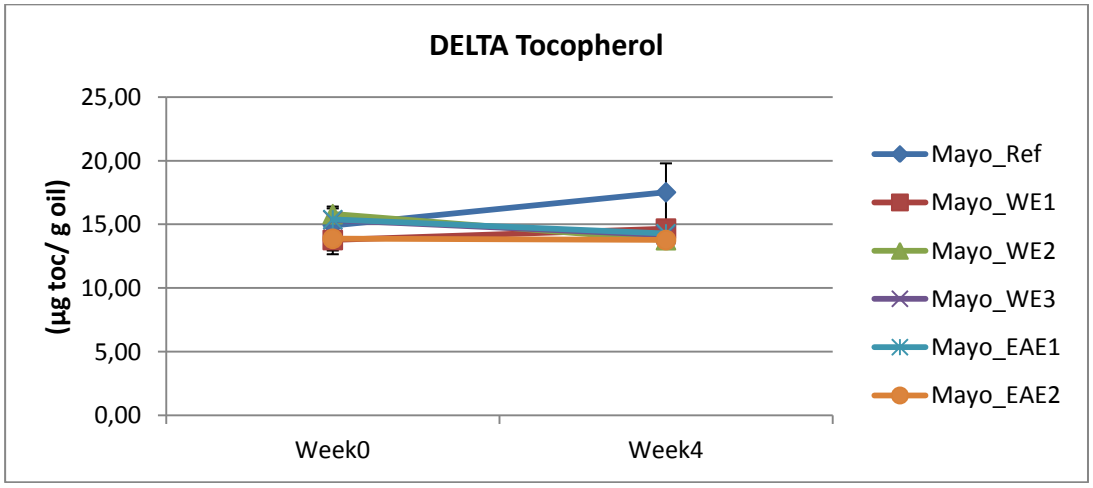

Figure 4.17: Delta tocopherol content of mayonnaise samples at week 0 and 4.

In summary, alpha and gamma tocopherol contents of fish oil enriched mayonnaise without added *F. vesiculosus* antioxidants was degraded faster than other samples

with *F. vesiculosus* extracts. Likewise, total tocopherol content of reference sample was decreasing while all the samples were not, which explains that *F. vesiculosus* extracts had an effect on protecting tocopherol content of mayonnaise samples.

4.4.5 Dynamic headspace

Identified volatile oxidation products were 1-penten-3-one, pentanal, 1-penten-3-ol, 3-methyl-1-butanol, 1-pentanol, hexanal, 2,4-heptadienal and nonanal in mayonnaise samples.

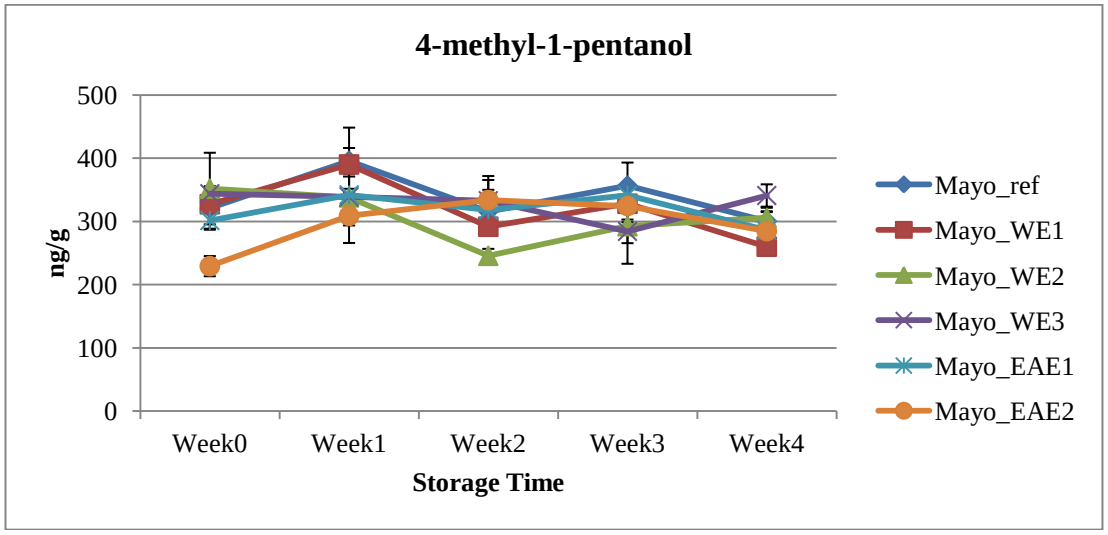

Figure 4.18: The 1-pentanol, 4-methyl content of the mayonnaises during 4 weeks of the storage (n=3).

4-methyl-1-pentanol was added to all samples as an internal standard and it was seen that Mayo_EAE2 has a low internal standard value at week 0 comparing to the other samples and storage times as shown in Figure 4.18. This low values were also seen for the other volatile oxidation compounds, it has to be concerned that this low values can be resulted from a mistake which might have done during the analysis or because of an error occured in the equipment during analysis.

Differences between amounts of 1-penten-3-one of Mayo_ref, Mayo_WE1 and Mayo_WE2 did not significantly change during 4 weeks storage time. Mayo_EAE2 was found significantly lower at week 0 comparing with the other storage time points. Mayo_EAE1 was found significantly higher at week 3 than other storage time points. When comparing 1-penten-3-one amounts of samples at week 0, Mayo_EAE2 was found significantly lower than the other samples. At week 1 and 2, Mayo_WE2 was found significantly lower than the other samples. At week 3, Mayo_WE3 was found significantly lower than the other samples. After the storage

experiment was done, no significant difference were found for all samples. In summary, 1-penten-3-one amounts did not change with a pattern during the 4 weeks storage experiment (Figure 4.19).

Sorensen et al. (2010) reported that 1-penten-3-one amount was determined as a value between 200-250 ng/g at the end of the 113 days of storage experiment (20°C) in fish oil enriched light mayonnaise samples (40% oil) which were prepared with 14% of fish oil (26% of rape seed oil) and egg yolk as an emulsifier. Larsen and Jacobsen (unpublished data) reported that the milk sample without *F. vesiculosus* was increased up to 25 ng/g, however, milk samples with *F. vesiculosus* extracts were found between 5 and 12 ng/g. Our results were found to be higher and without a clear pattern.

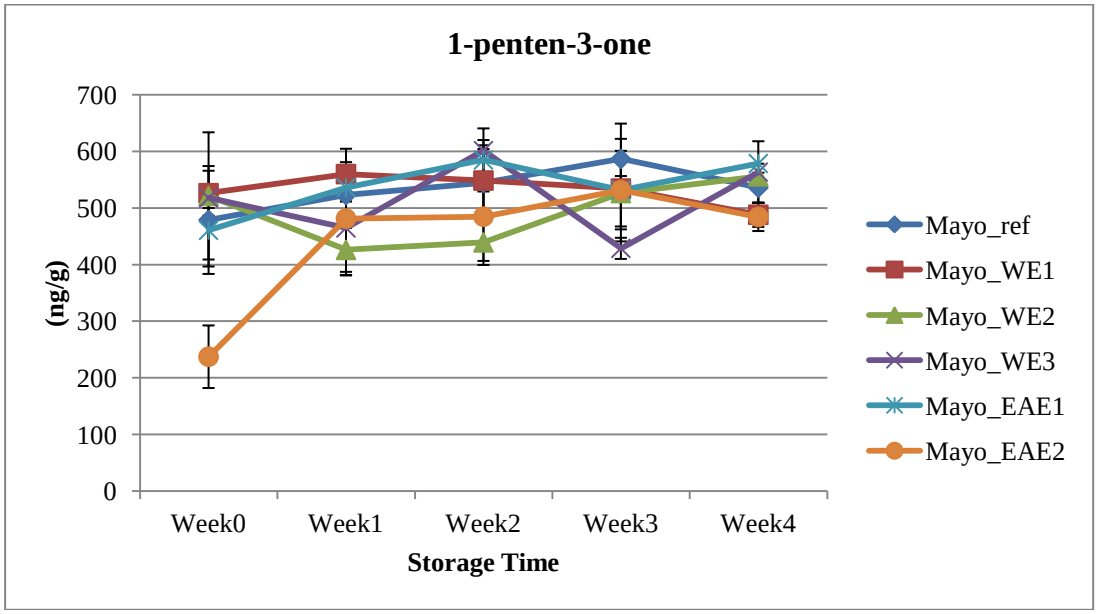

Figure 4.19: The 1-penten-3-one content of the mayonnaises during 4 weeks of the storage (n=3).

As shown on the Figure 4.20, pentanal amount of Mayo_ref was significantly increased every week during the storage experiment. When comparing the changes between weeks, no significant change was observed at the beginning of the experiment (week 0). After one week, Mayo_ref, Mayo_WE3 and Mayo_EAE2 had significantly lower value than the other samples. After week 2, Mayo_ref and Mayo_WE3 were found to be significantly lower than the other samples. After 3rd week, Mayo_WE3 was significantly lower than the other samples. At the end of the storage experiment, Mayo_WE3 was significantly lower and Mayo_ref was significantly higher than the other samples. This showed that mayonnaise sample

with the water extracts (conc. 2 g/kg) of *F. vesiculosus* increased the oxidative stability comparing to the reference sample.

According to the results reported by Jacobsen et al. (2001), concentrations of pentanal were changing between 20 to 130 ng/g in 16% fish oil enriched mayonnaise without using antioxidant during 4 weeks of storage period (pH 3.8 which is similar to our samples pH). In this study pentanal concentrations of 16% fish oil enriched mayonnaise sample without *F. vesiculosus* extracts addition are changing between 10 and 65 ng/g. Mayonnaise samples in the same properties but with the concentration of 2 g/kg water extracts of *F. vesiculosus* had pentanal concentration changing between 8 to 44 ng/g.

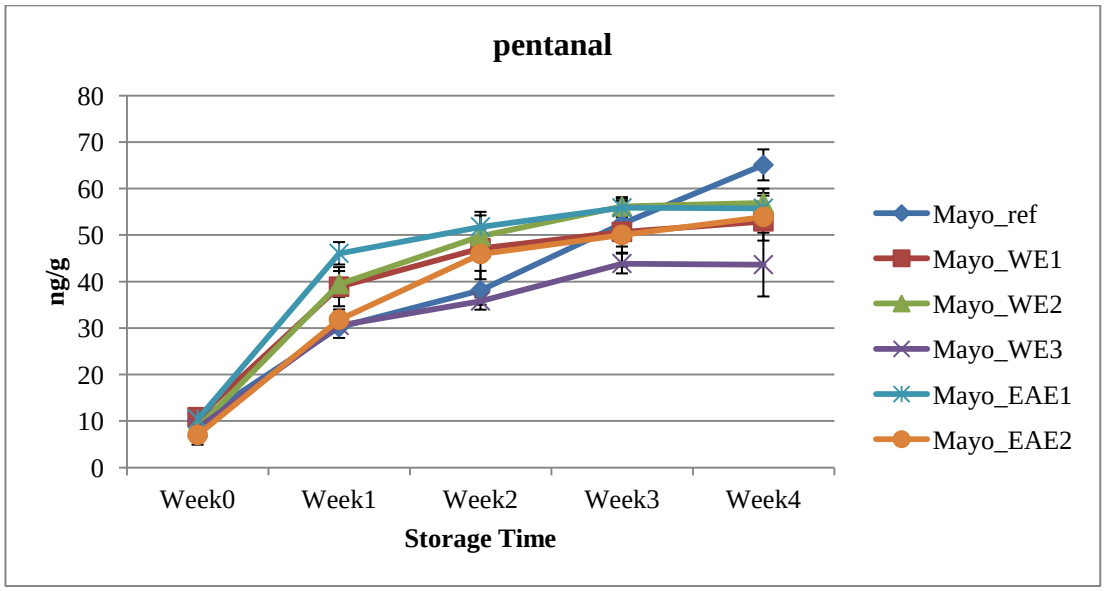

Figure 4.20: The pentanal content of the mayonnaises during 4 weeks of the storage (n=3).

1-penten-3-ol amount of Mayo_ref, Mayo_WE1 and Mayo_WE2 samples were found to be significantly increased after 2nd,3rd and 4th weeks of the storage experiment compared to week 0 and 1. On the other hand, amount of 1-penten-3-ol for Mayo_WE3 was not changed significantly during the storage experiment. Mayo_EAE1 was increased significantly after 3rd and 4th weeks compared to week 0, 1 and 2; Mayo_EAE2 was increased after 4th week compared to week 0, 1, 2and 3. No significant difference was observed between the samples at week 0 and 1. After week 2, 3 and 4, 1-penten-3-ol content of Mayo_WE3 was determined as significantly lower than the other samples, followed by Mayo_EAE2 and Mayo_EAE1, respectively (Figure 4.21). At the end of the storage experiment, the ranking considering oxidative stability of the samples was found to be in the order of

Mayo_WE3 > Mayo_EAE2 > Mayo_EAE1 > Mayo_WE2 > Mayo_WE1 > Mayo_ref. This indicated that water extract (conc. 2 g/kg) had significantly high oxidative stability in fish oil enriched mayonnaise comparing to all the samples. And all samples including *F. vesiculosus* extracts showed significantly higher oxidative stability comparing to reference sample.

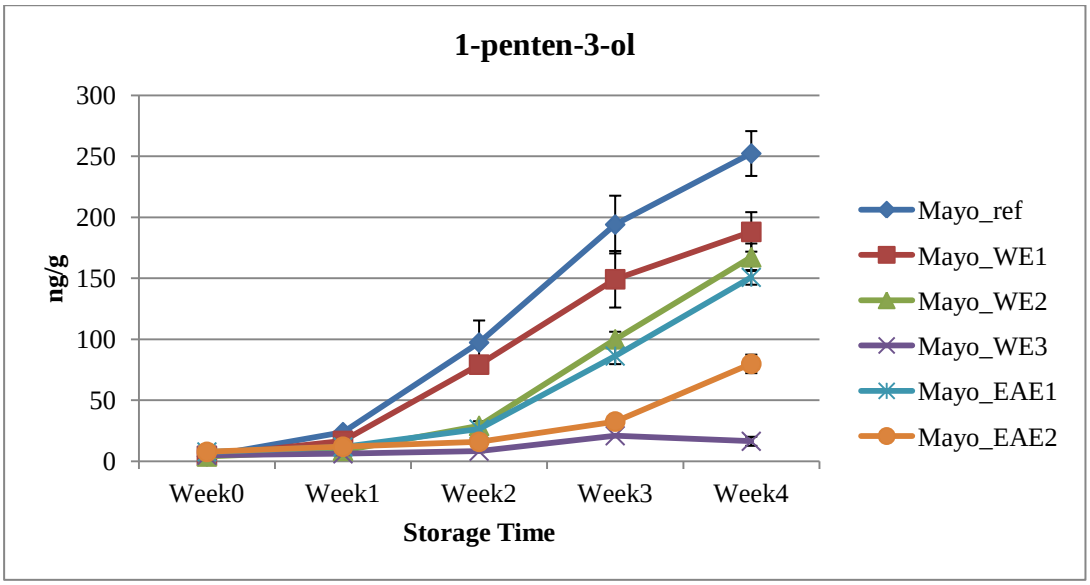

Figure 4.21: The 1-penten-3-ol content of the mayonnaises during 4 weeks of the storage (n=3).

As shown at the Figure 4.22, 3-methyl-1-butanol content of Mayo_ref and Mayo_WE1 samples were significantly increased after week 2 and week 3. 1-butanyl,3-methyl content of Mayo_WE2 sample was significantly increased after 3rd week. 1-butanyl,3-methyl amount of Mayo_WE3 sample was not increased significantly during storage experiment. Mayo_EAE1 sample was significantly increased after week 3 and 4. Mayo_EAE2 sample was significantly increased only after week 4. When comparing the changes between samples, no significant difference were found at week 0 and 1. At week 2, 1-butanyl,3-methyl content of Mayo_ref and Mayo_WE1 were significantly higher than the others. At week 3, 1-butanyl,3-methyl content of Mayo_WE3, Mayo_EAE1 and Mayo_EAE2 were found significantly lower than other samples. Mayo_WE1 and Mayo_WE2 were also significantly lower than the referans mayonnaise sample. At the end of the storage experiment, 1-butanyl,3-methyl content of Mayo_WE3 and Mayo_EAE2 were found significantly lower than other samples. 1-butanyl,3-methyl content of Mayo_WE1, Mayo_WE2 and Mayo_EAE1 samples were found significantly lower than referans sample.

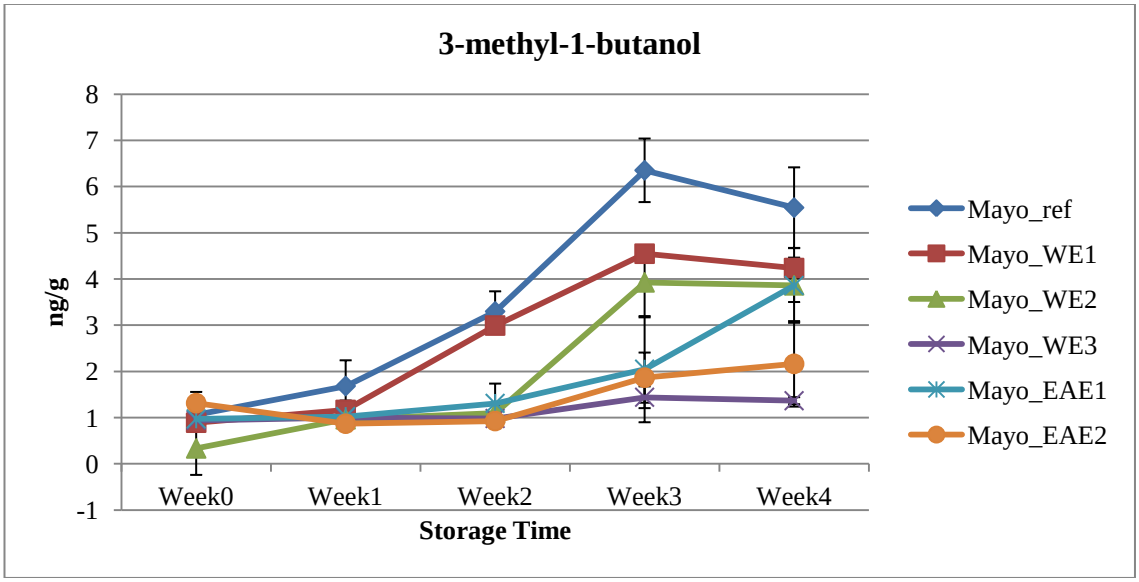

Figure 4.22: The 3-methyl-1-butanol content of the mayonnaises during 4 weeks of the storage (n=3).

1-pentanol content of Mayo_ref was significantly decreased after week 4 as shown in the Figure 4.23. 1-pentanol content of Mayo_WE1 was significantly decrease after week 2 and week 4. 1-pentanol content of Mayo_WE2 was found significantly low at week 2 comparing to the starting point of the storage experiment. 1-pentanol content of Mayo_WE3 was significantly higher at week 2 and lower at 3 comparing with other weeks. No significant differences were observed for 1-pentanol content of Mayo_EAE1 during the storage experiment. Likewise, 1-pentanol content of Mayo_EAE2 showed no significant difference except for week 0. Acording to these data, there was no clear pattern observed.

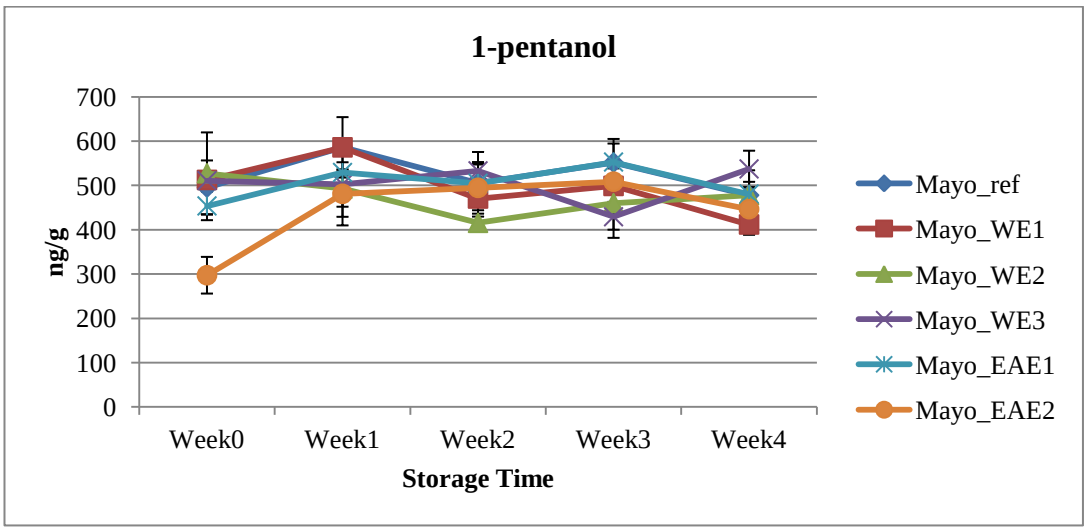

Figure 4.23: The 1-pentanol content of the mayonnaises during 4 weeks of the storage (n=3).

As shown on the Figure 4.24, hexanal did not show a clear pattern.

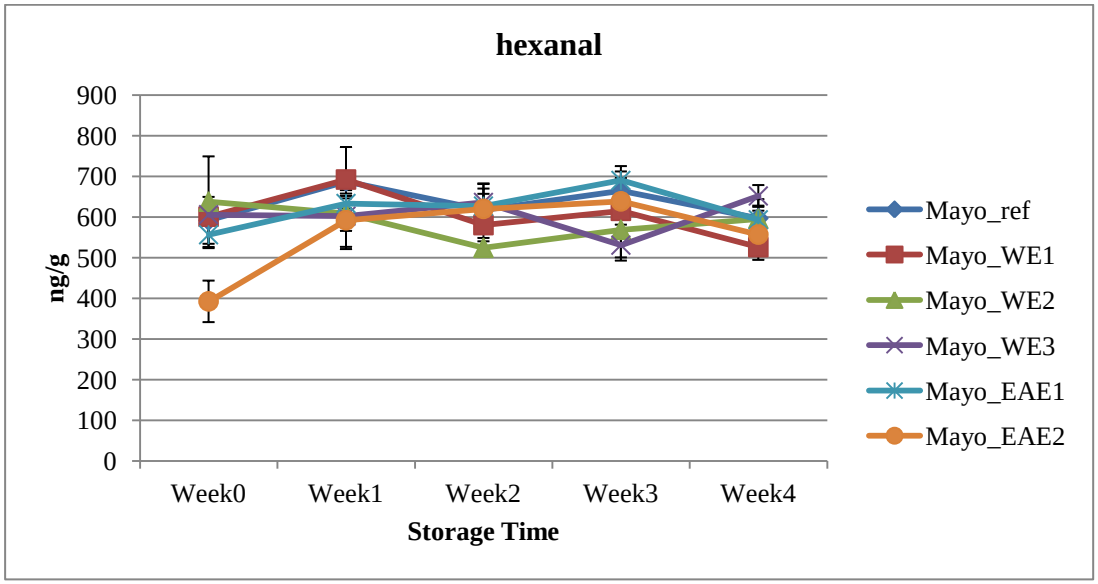

Figure 4.24: The hexanal content of the mayonnaises during 4 weeks of the storage (n=3).

As shown on the Figure 4.25, 2,4-heptadienal does not indicate a clear pattern. According to Jacobsen et al. (2001), 2,4-heptadienal increased from 36 to 180 ng/g in 16% fish oil enriched mayonnaise without antioxidant during 4 weeks of storage period (pH 3.8 which is similar to our samples pH). However, our results have higher values and does not show a clear pattern.

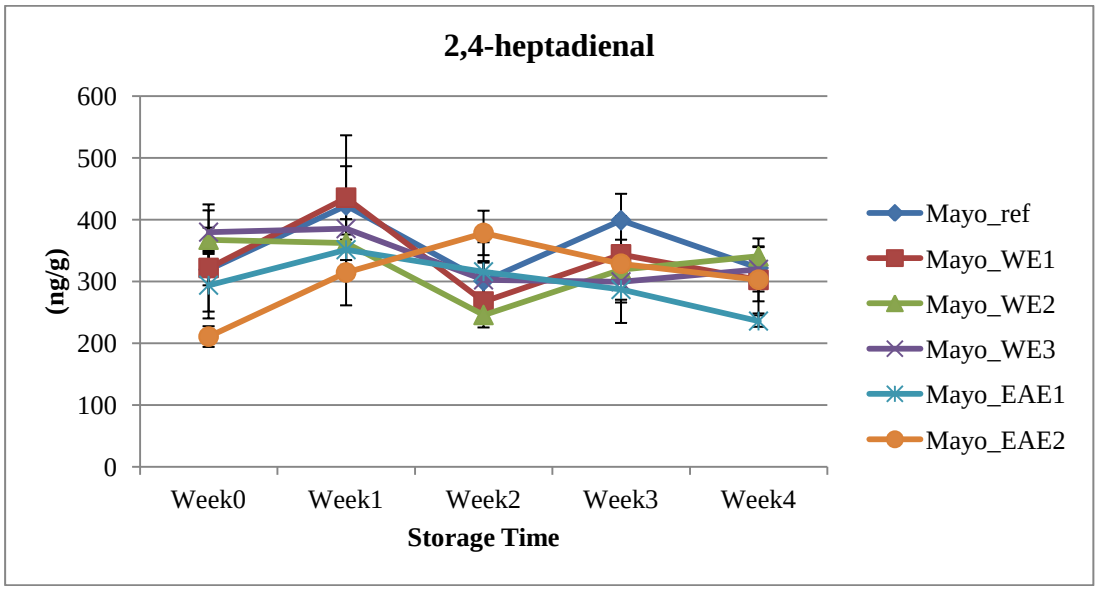

Figure 4.25: The 2,4-heptadienal content of the mayonnaises during 4 weeks of the storage (n=3).

As shown on the Figure 4.26, nonanal content of Mayo_EAE2 has higher values whereas there do not seem to be any clear pattern between the other samples.

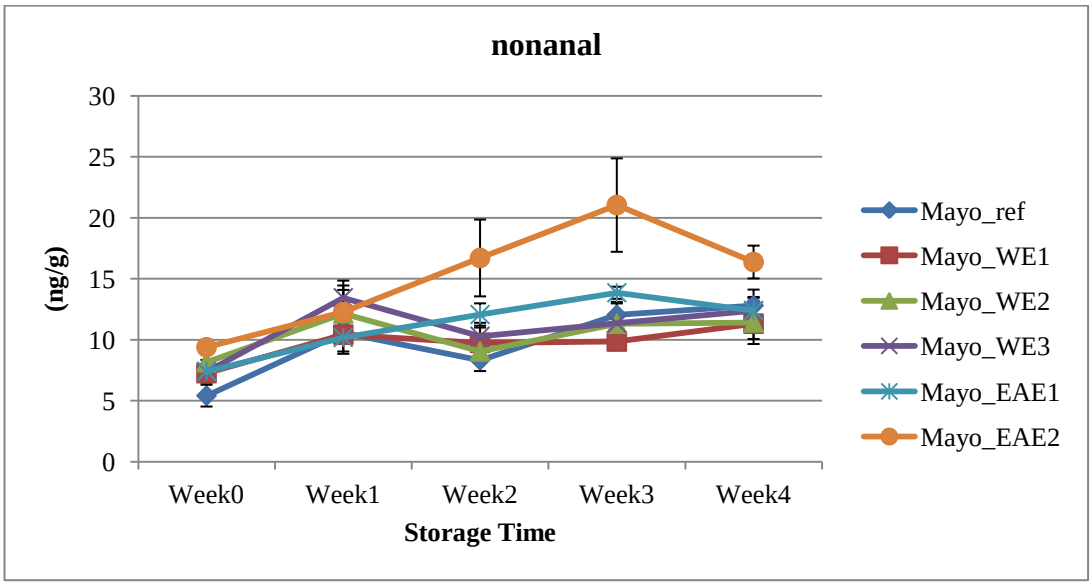

Figure 4.26: The nonanal content of the mayonnaises during 4 weeks of the storage (n=3).

4.4.6 Sensory Analysis

Sensory analysis was performed by 3 trained panelists considering fishy/rancid, acidity, other such as metallic odors and consistency by a help of a spoon. Samples were taken at the beginning of the storage experiment and at the end of the storage experiment. Since evaluation was done by each panelist and then decided on a score together with a discussion between panelists, there is no standard deviation and performed statistic analysis.

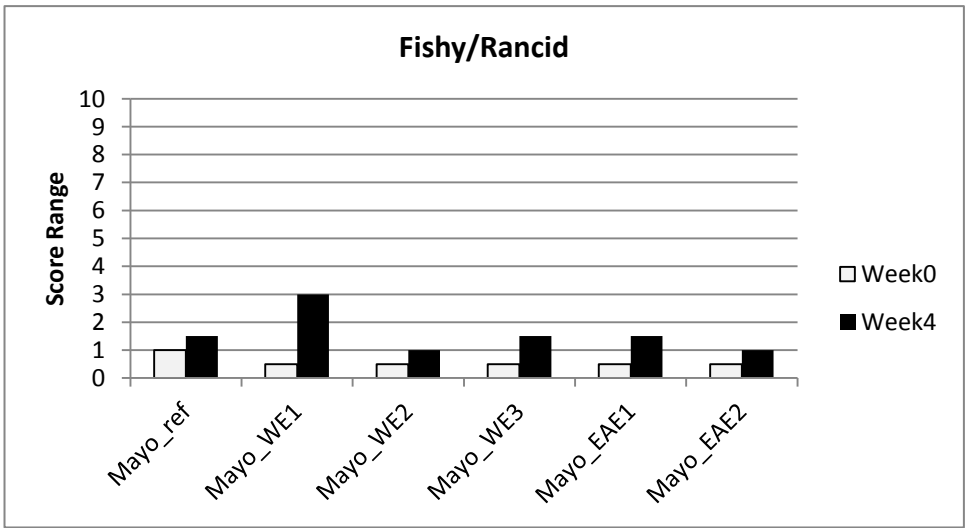

Figure 4.27: The sensory evaluation of mayonnaise fishy/rancid odor at week 0 and 4.

As shown at the Figure 4.27, fishy/rancid odors of samples were determined between 0.5 and 1 in the score range of 0 to 10 at the beginning of the storage experiment. At the end of the storage experiment, results for fishy/rancid odors were observed between 1 to 3 in the score range of 0 to 10. Biggest increase between the samples was observed for Mayo_WE1 sample. When sensory anaylsis results were compared with PV results, it was seen that Mayo_WE1 had the second highest value at the end of the storage experiment and also was found high in terms of fishy/rancid odor. However, peroxides are odorless, this might be due to the the high content of secondary oxidation products such as 1-penten-3-ol, pentanal and 3-methyl-1-butanol.

Jacobsen et al. (2000) reported that off-flavor development was slightly higher in mayonnaises with larger droplets than smaller ones. When out results were observed regarding this argument, Mayo_EAE2 and Mayo_WE3 had higher droplet size comparing to others and they showed lower fishy/rancid odor comparing to Mayo_WE1 which had small droplet size comparing to Mayo_EAE2 and Mayo_WE3.

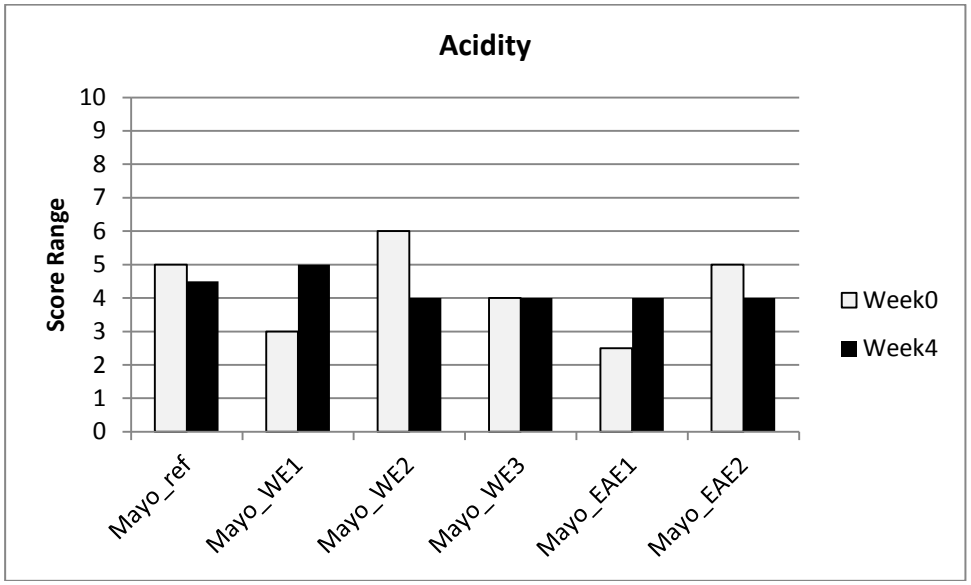

Figure 4.28: The sensory evaluation of mayonnaise acidity at week 0 and 4.

At the beginning of the storage experiment, acidic odor of the samples were determined between 2.5 and 6 out of 10. After 4 weeks, results were determined between 4 and 5 out of 10. Mayonnaise samples had pH values changing between 4.30 and 4.38. Even though the range of the determined acidity values at the beginning were larger than the week 4, it looked like at the end of the storage experiment there was no difference indicated except for Mayo_WE1 with a small

difference as shown in the Figure 4.28. This explains that extracts did not show an important effect on the acidity.

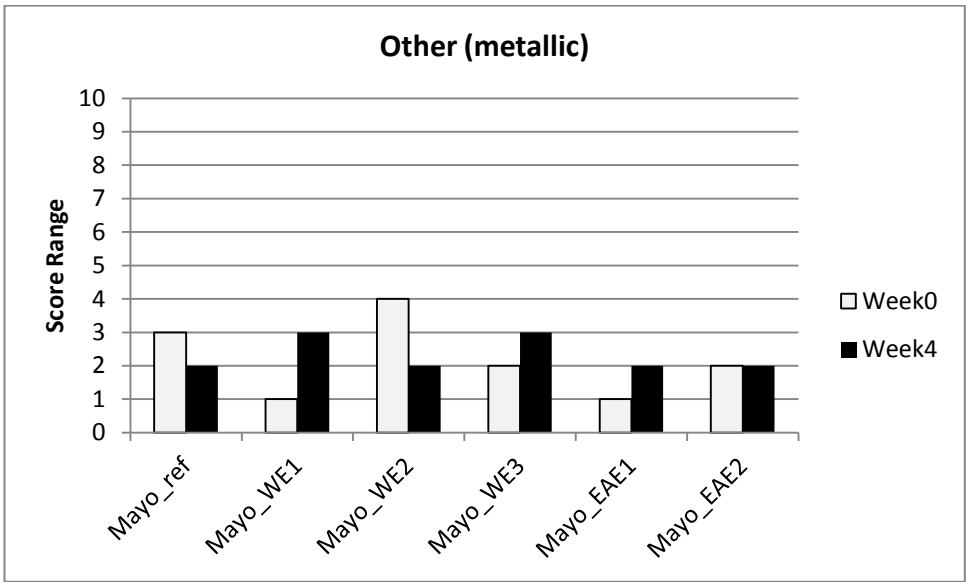

Figure 4.29: The sensory evaluation of mayonnaise other (metallic) odors at week 0 and 4.

Other odors from the samples were mainly considered as a metallic odor. At the beginning of the storage experiment, metallic odor score of the samples were found between 1 and 4 out of 10. At the end of the storage experiment, results for the samples were determined between 2 to 3 out of 10 (Figure 4.29).

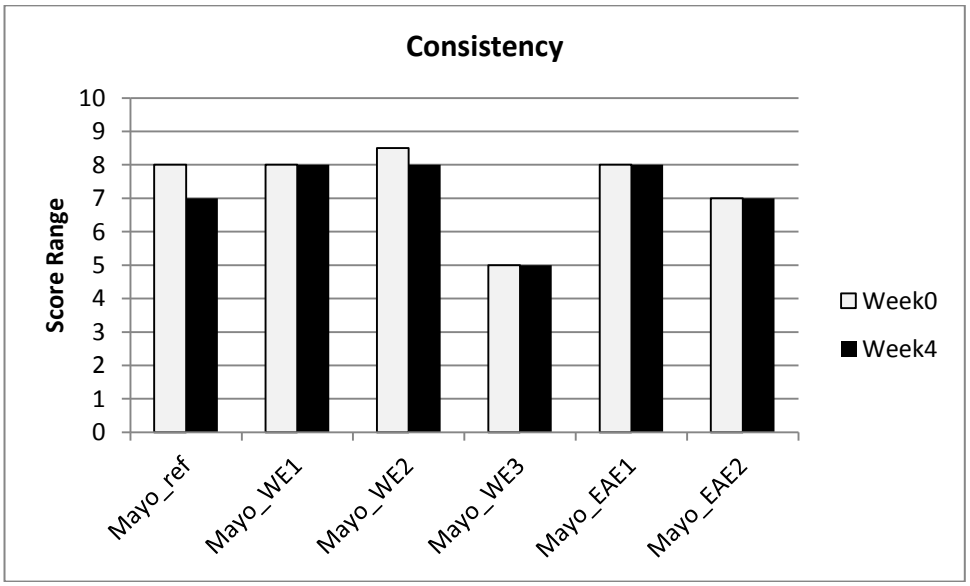

Figure 4.30: The sensory evaluation of mayonnaise consistency at week 0 and 4.

Consistency results of samples were determined with a help of a spoon and found between 5 to 8.5 out of 10. At the end of the storage experiment, results were between 5 and 8 out of 10. Mayo_WE3 had lower consistency than the other

samples, it may be because of mixing salt and sugar after mixing oil and water phase together instead of mixing them first in water phase. When comparing values according to their storage times, it was observed that not much difference were seen between week 0 and 4 (Figure 4.30).

5. CONCLUSION

The aim of the study was to investigate the effects of *F. vesiculosus* extracts by identifying phlorotannins, assessing antioxidant activity, applying *in vitro* gastrointestinal digestion method and adding *F. vesiculosus* extracts in fish oil enriched mayonnaise for 4 weeks storage experiment. According to the results, phlorotannin content of *F. vesiculosus* extracts were found to be higher than many fruits and plants which are known for their high phenolic content. Metal chelating activity of water extracts of *F. vesiculosus* was higher than that of the ethylacetate extracts of *F. vesiculosus*. In contrast, DPPH scavenging activity and reducing power of ethylacetate extracts of *F. vesiculosus* were higher than those of the water extracts. After *in vitro* gastrointestinal digestion of the extracts, bioavailability of the phlorotannin content of the extracts were found to be slightly lower compared to the phenols recovered from e.g. raspberry. However, after *in vitro* gastrointestinal digestion, both digested water and ethylacetate extracts showed low DPPH radical scavenging activity but high metal chelating activity. Reducing power was found to be high only for the digested ethylacetate extract of *F. vesiculosus,* not for the water extract. Preliminary characterization of four phlorotannins was done by LC-MS, and fucophloroethol A, fucodiphloroethol G, fucotriphlorethol A, trifucodiphlorethol A were identified in purified ethylacetate extracts and some of the fractions. Both water and ethylacetate extracts showed an effect on oxidative stability in fish oil enriched mayonnaise. Droplet size was increased with the increasing concentration of both extracts. Mayonnaises with smaller droplet sizes were oxidised faster at the initial part of the storage period than the ones with larger droplet sizes. Water extract of *F. vesiculosus* (conc. 2 g/kg) showed a slow increase in peroxide value compared to other samples during 4 weeks of storage experiment. Ethylacetate extract of *F. vesiculosus* (conc. 1 g/kg) had a similar behavior with a higher increase compared to *F. vesiculosus* (conc. 2 g/kg). EPA and DHA loss were prevented by the addition of *F. vesiculosus* water and ethylacetate extracts during 4 weeks storage. Mayonnaise samples with *F. vesiculosus* water and ethylacetate extracts showed slower degradation comparing to the reference samples. Volatile secondary oxidation products such as pentanal, 1-penten-3-ol, 3-methyl-1-butanol was found to be decreased by *F. vesiculosus* extracts especially by water extract of *F. vesiculosus* with the 2 g/kg concentration.

6. PERSPECTIVES

Fractionation of extracts should be improved by trying different solvent systems which change from polar to apolar and also pH values to be able to separate different phlorotannins into different fractions. Thus, it will be easier to see the effects of phlorotannins individually. Characterization of phlorotannins should be done using LC-MS/MS and MS techniques should be developed in order to identify the compounds responsible for the antioxidant activity. Antioxidant activity of these fractions can be investigated and the ones which show better activity can be added into fish oil enriched mayonnaise to evaluate their effects on oxidative stability. *In vitro* gastrointestinal method needs to be optimized considering the amount of *F. vesiculosus* extracts added into the system in order to find the right amount to get more clear results. Simple sensory analysis results were done by considering only odors and it was acceptable compared to reference mayonnaise considering texture, color and odor properties. However, sensory analysis should be repeated with more panelists and the number of evaluated criterias can be increased to get a better understanding on flavor, aroma and odor changes in the product during the storage. Higher concentration of water and ethylacetate extracts can be added into the fish oil enriched mayonnaise and analysed to see their behaviours on tocopherol content and omega-3 fatty acids (EPA and DHA) , more clearly. Since *F. vesiculosus* includes high amounts of iodine, iodine content of extracts should be analysed in order to determine the intake when it is consumed regularly in a food product (Norman et al., 1987). Another important factor is its heavy metal content, it sould also be considered in order to know how much heavy metal does it include in extracts (Walkiw and Douglas, 1974).

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CURRICULUM VITAE

Name Surname: Betül Yeşiltaş

Place and Date of Birth: Istanbul, 15.12.1988

Address: Ethem efendi cad. 2. orta sok.Bakibey apt. 64/8 Erenköy

E-Mail: betulyesiltas@gmail.com

B.Sc.: Food Engineering, Istanbul Technical University

Professional Experience and Rewards:

- Danone Baby Nutrition – Numil Food R&D Departmant Portfolio Manager Assistant (9 months)
- Istanbul Technical University, Food Engineering Department, European Union Project (ATHENA)-Project Assisstant (10 months)

List of Publications and Patents:

- Yeşiltaş, B.,Capanoglu, E., Firatligil-Durmus, E., Sunay, A.E., Samanci, T., Boyacioglu, D., 2013. Investigating The *in-vitro* Bioaccessibility of Propolis and Pollen Using a Simulated Gastrointestinal Digestion System, *Journal of Apicultural Research* (Accepted)
- Yeşiltaş, B., Çapanoğlu, E., Fıratlıgil, E., Boyacıoğlu, D., 2012 March (19-21), *1st International Conference on Food Digestion*, Cesena-Italy. Investigating the Antioxidant Potential and in-vitro Bioavailability of Propolis (Poster Presentation)
- Yeşiltaş, B., Çapanoğlu, E., Fıratlıgil, E., Boyacıoğlu, D., 2012 May (3-4), *3rd Food Safety Congress*, Istanbul, Turkey. Investigating the antioxidant capacity of pollen samples (Poster Presentation)
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PUBLICATIONS/PRESENTATIONS ON THE THESIS

- Larsen, D.B., Yeşiltaş, B., Kristinsson, H., Jónsdottir, R., Jacobsen, C., 2013. Antioxidant Effect of Seaweed Extracts in *in Vitro* and Food Emulsion Systems Enriched with Fish Oil. *American Oil Chemists' Society Annual Meeting*, April 29, 2013, Montreal, Canada.